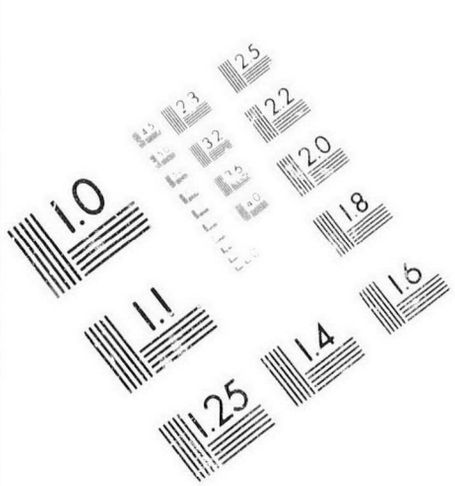

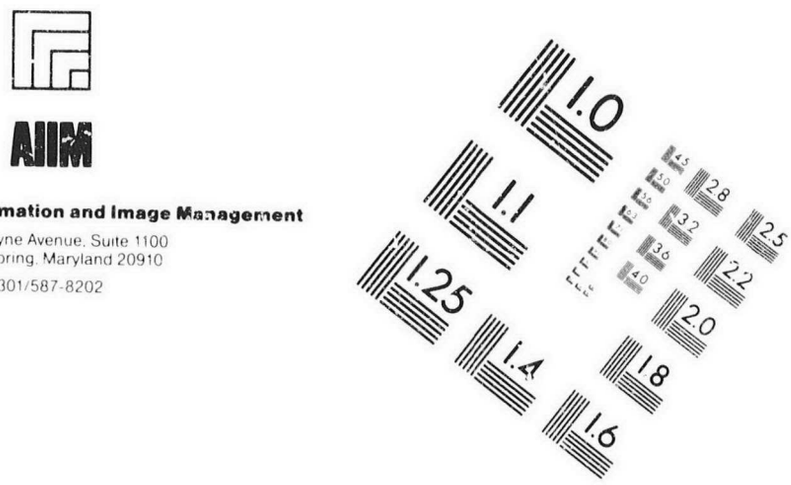

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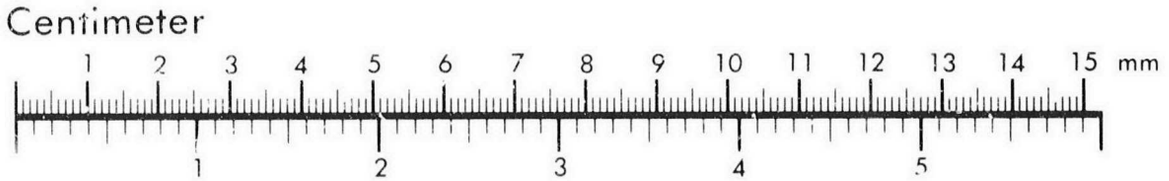

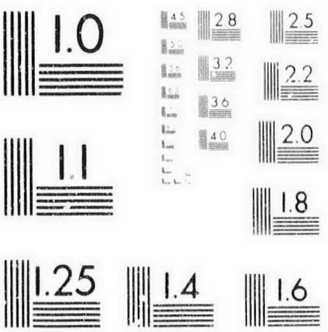

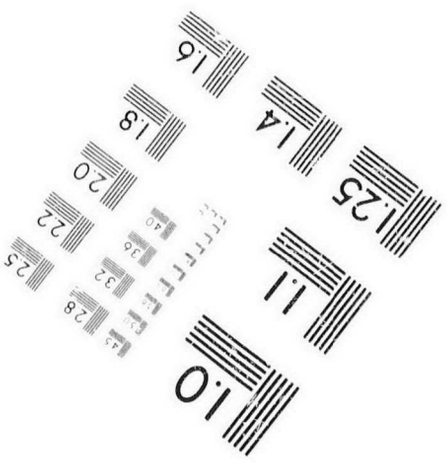

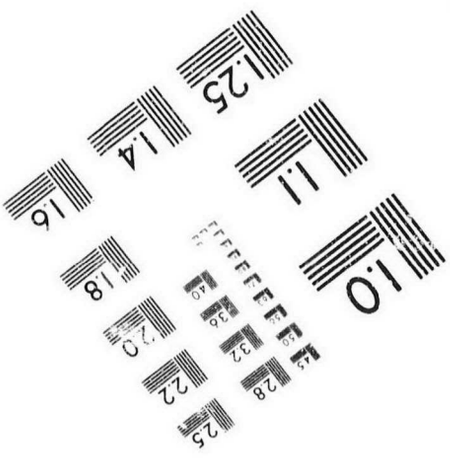

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Document Title		
1,6-HEXAMETHYLENE DIISOCYANATE, TOLUENE DIISOCYANATE LUNG SENSITIZATION IN GUINEA-PIGS FOLLOWING INTRADERMAL OR INHALATION INDUCTION, W/TSCA HLTH & SFTY STUDY CVR SHT DATED 5/20/97		
Chemical Category		
2,4/2,6-TOLUENE DIISOCYANATE (26471-62-5); *		

OFFICE OF TOXIC SUBSTANCES

REV. 7/27/82

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☐ **CHECK IF THIS PAGE CONTAINS CONFIDENTIAL BUSINESS INFORMATION (CBI)**

Clearly mark the confidential information with bracketing and check the box in the appropriate section (☐*Contains CBI*). Submit a sanitized cover sheet with CBI deleted. Mark the sanitized copy, "Public Display Copy" in the heading.

1.0 SUBMISSION TYPE ☐*Contains CBI*

X 8(d) ☐ 8(e) ☐ FYI ☐ 4 ☐ OTHER: Specify_____

X - Intial Submission ☐ Follow-up Submission **X =Final** Report Submission

Previous EPA Submission Number or Title if update or follow-up: Docket Number, if any: #

☐ continuation sheet attached

2.1 SUMMARY/ABSTRACT ATTACHED (may be required for 8(e): optional for §4, 8(d) & FYI) ☐ YES **X** NO	**2.2 SUBMITTER TRACKING NUMBER OR INTERNAL ID** Cert# P 917006693 97-12-4	**2.3 FOR EPA USE ONLY**

3.0 CHEMICAL/TEST SUBSTANCE IDENTITY ☐*Contains CBI*

Reported Chemical Name (specify nomenclature if other than CAS name):

CAS#: 822-06-0 and 26471-62-5 1,6-Hexamethylene diisocyanate and 2,4/2,6-Toluene Diisocyanate

Purity _____%

☐ Single Ingredient

☐ Commerical/Tech Grade

X -Mixture *Trade Name:* Desmodur H, HDI and 2,6/2,4-TDI *Common Name:*

	CAS Number	*NAME*	*% WEIGHT*
Other chemical(s) present in tested mixture			

Contains No CBI

☐ continuation sheet attached

4.0 REPORT/STUDY TITLE ☐*Contains CBI*

1,6-Hexamethylene diisocyanate Toluene Diisocyanate - Lung Sensitization in Guinea-pigs following Intradermal or Inhalation Induction Report # 25460

☐

5.1 STUDY/TSCATS INDEXING TERMS

[CHECK ONE]

HEALTH EFFECTS (HE): X ENVIRONMENTAL EFFECTS (EE):_____ ENVIRONMENTAL FATE (EF):

5.2 STUDY/TSCATS INDEXING TERMS (see instructions for 4 digit codes)

STUDY TYPE: _____	SUBJECT ORGANISM (HE, EE only): GUIN	ROUTE OF EXPOSURE (HE only):_____	VEHICLE OF EXPRE (HE)_____
Other: ***Lung S ...tization*** *Other:*		*Other:*	*Other:*

6.0 REPORT/STUDY INFORMATION ☐*Contains CBI* - Study is GLP **X**

Laboratory Bayer AG Tox Lab Report/Study Date **9/24/96**

Source of Data/Study Sponsor (if different than submitter) Bayer Number of pages : 9

☐ continuation sheet attached

7.0 SUBMITTER INFORMATION ☐*Contains CBI*

Submitter: **Francis J. Rattay** Title: **Manager, Reg. Affairs** Phone: (412) **777-7471**

Company Name: **Bayer Corporation** Company Address: **100 Bayer Road**

Pittsburgh, PA 15205-9741 Submitter Address (if different): _____

Technical Contact: **Francis J. Rattay** Phone: (412)**777-7471**

☐ continuation sheet attached

8.0 ADDITIONAL/OPTIONAL STUDY COMMENTS ☐*Contains CBI*

Study indicates 2914 pages, however we are submitting all that we have in our possession.

☐ continuation sheet attached

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Submitter Signature: F.J. Rattay (VMK) **Date:** 5/20/97

Page 1 of 1

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BAYER AG
DEPARTMENT OF TOXICOLOGY
FRIEDRICH-EBERT-STR. 217-333
D - 42096 WUPPERTAL

Report-No.: 25460

Date: 24.09.1996

1,6-HEXAMETHYLENE DIISOCYANATE
TOLUENE DIISOCYANATE
(Desmodur H and Desmodur T80)

Lung Sensitization in Guinea-pigs following Intradermal or Inhalation Induction

by

PD Dr. J. Pauluhn

Study Numbers: T3060304 / T3060700

This page is intentionally left blank for the purpose of submitting administrative information that is required by regulations promulgated by various countries.

GOOD LABORATORY PRACTICE STATEMENT

This study was conducted in compliance with the OECD Principles of Good Laboratory Practice (GLP) and to the Principles of Good Laboratory Practice (GLP) according to Annex 1 ChemG (Bundesanzeiger No. 42a of March 2, 1983 and Bundesgesetzblatt, Part I of July 29, 1994), except that this report has not been audited by Quality Assurance.

Date: August 8, 1996

PD Dr. J. Pauluhn D.A.B.T.
Board Approved Toxicologist (DGPT)
Study Director

1. TABLE OF CONTENTS

2. QUALITY ASSURANCE STATEMENT

Test Substance: **1,6-HEXAMETHYLENE DIISOCYANATE**
HDI and TDI

Study No.: **T3060304 / T3060700**

The study was audited by Quality Assurance on the dates given below. Audit reports have been submitted in writing to the study director and, if necessary, also the laboratory management, or other persons affected.

Date of audit	**Date of report to study director/management**
T3060304:	
Oct. 04, 1995 (study plan)	Oct. 05, 1995
Oct. 04, 1995	Oct. 04, 1995
Nov. 14, 1995	Nov. 14, 1995
Dec. 11, 1995	Dec. 11, 1995
Dec. 12, 1995	Dec. 12, 1995
T3060700:	
Jan. 24, 1996 (study plan)	Jan. 24, 1996
Feb. 20, 1996	Feb. 20, 1996
Feb. 21, 1996	Feb. 21, 1996
Mar. 07, 1996	Mar. 07, 1996

This study report was not audited by Quality Assurance.

Quality Assurance Unit
PH-QA-C/GLP, Bayer AG

Date: Aug. 07 1996

Responsible: Dr.R.Rauchschwalbe

3. SIGNATURES

Study director: _____ August 8, 1996
PD Dr. J. Pauluhn Date

Characterization of atmospheres: _____ Aug. 30, 1996
Dr. W. Rüngeler Date

Head of Institute: _____ Sept. 13, 96
Prof. Dr. E. Löser Date

4. SUMMARY

A lung sensitization study with 1,6-HEXAMETHYLENE DIISOCYANATE (HDI) and TOLUENE DIISOCYANATE (TDI) was performed using guinea-pigs of the Dunkin-Hartley Pirbright-White (DHPW) strain. A standard approach was used that included either three intradermal injections (one per day) or 5x3 hrs inhalation exposures, including one additional intradermal injection, followed by inhalation challenge with the hapten, acetylcholine and conjugate by inhalation.

Study design: Groups of eight female guinea-pigs were intradermally induced once per day on days 0, 2, and 4 (injection volume: 100 µl/0.3% solution in desiccated corn oil) and one additional groups of animals were exposed for five consecutive days (days 0 - 4) by inhalation (duration of exposure: 3 hrs/day) to average concentrations of HDI and TDI (vapor) of 27.4 mg/m³ air and 51.4 mg/m³ air, respectively. The latter groups of animals received an additional single intradermal induction on day 0 so as described above. Control animals received the vehicle alone (intradermally) under otherwise identical conditions (no inhalation exposure). During the recovery period (starting on day 21) a hapten-challenge (target concentrations: approximately 0.5 mg hapten/m³ air) was performed (challenge duration: 30 min). One day before and one day after the hapten-challenge an acetylcholine bronchoprovocation challenge (stepped concentrations in steps of 0.1%, 0.2%, 0.4% and 0.8%, w/v; duration of each 15-min) was performed. Following day 28 all guinea pigs were challenged again with the respective guinea pig serum albumin (GPSA) conjugate of the hapten (mean concentration: approximately 50 mg/m³ air). During and after challenge exposures immediate-onset respiratory reactions were evaluated by measurement of respiratory rate, tidal volume, respiratory minute volume, inspiratory and expiratory times, and peak expiratory flow rate. Additional parameters were derived mathematically. In some of the groups also measurements for delayed-onset responses were incorporated.

One day after the GPSA-conjugate challenge, animals were sacrificed, and the lungs, including trachea and lung associated lymph nodes, were examined histopathologically (exception: groups sensitized intradermally with TDI). The weight of the excised lungs was

determined. At sacrifice blood was sampled for serological examinations (exception: groups sensitized intradermally with TDI).

Summary of results: Following induction, inflammatory skin reactions were observed. Also during the inhalation induction, signs indicative of respiratory tract irritation occurred. During or following hapten-challenges, the incidence of immediate-onset type respiratory reactions were roughly the same in all groups whereas during or following conjugate-challenges immediate-onset respiratory reactions occurred in a higher incidence in the HDI and TDI sensitized groups when compared to the control groups. The comparison of both routes of induction demonstrate that the incidence of responding animals was not appreciably different when additional inhalation induction exposures were made. The acetylcholine bronchoprovocation challenge demonstrated that previous inhalation induction exposures to TDI, but not HDI, evoked a conspicuous nonspecific bronchial hyperreactivity. The histopathological investigations revealed inflammatory responses in those groups receiving inhalation induction exposures (bronchiolitis). Independent on the route of induction in both the HDI and TDI groups evidence of a specific airway eosinophilia and eosinophil infiltration into lung associated lymph nodes, a hallmark of allergic airway hyperresponsiveness, was observed. The serological investigation revealed a marked increase in anti-HDI/TDI IgG_1-antibody titres.

Assessment: When animals that were sensitized intradermally or by inhalation and were subsequently challenged by inhalation with the respective hapten HDI or TDI no conclusive immediate-onset responses were observed. As a result of challenge with the respective conjugate of the haptens conclusive immediate-onset responses occurred. Additional evidence of a lung sensitizing potential was provided by the histopathological examinations which revealed an increased eosinophilia of airways and lung associated lymph nodes as well as production of specific IgG_1-antibody. Therefore, this study provides clear evidence that HDI and TDI are a respiratory sensitizer in the guinea pig bioassay. It does not appear, in turn, that the combined intradermal and inhalation induction produces synergistic effects. These findings lend support the conclusion that successful induction and elicitation of allergic respiratory hypersensitivity can be achieved either by intradermal and by inhalation induction exposures. The first, however, is easier to standardize and less prone to experimental artifacts.

5. INTRODUCTION

A lung sensitization study with HDI was performed using guinea-pigs of the Dunkin-Hartley Pirbright-White (DHPW) strain. TDI, a known human respiratory tract sensitizing agent, was used as reference compound. The principles of this experimental model have been published elsewhere (Botham *et al.*, 1989; Pauluhn and Eben, 1991; Pauluhn, 1994b)). Published evidence suggests that an *intradermal-induction* and *inhalation-challenge* protocol appear to be less susceptible to confounding factors such as irritation-induced airway hyperreactivity, when compared with the *inhalation-induction inhalation-challenge* protocol (ECETOC, 1993).

In order to investigate whether the test substances have any potential to induce specific or non-specific airway hyperreactivity a novel, combined intradermal/inhalation sensitization approach was attempted. The primary objective of this study was to demonstrate that this model serves the purpose for classification of (di)isocyanates as well as to examine whether combined induction regimens would even increase the sensitivity of this bioassay.

This study was conducted during the periods specified below at the following testing facility: Institute of Toxicology - Industrial Chemicals of the Bayer AG Fachbereich Toxikologie in D-42096 Wuppertal, Friedrich-Ebert-Strasse 217 - 333.

Study no. : **T3060304 / HDI**
Duration of study : February 2, 1996 to March 7, 1996

Study no. : **T3060700 / TDI**
Duration of study : Oktober 2, 1995 to December 12, 1995

6. RESPONSIBILITIES

Air conditioning/air make-up ..... Dipl. Ing. G. Strietholt
Archiving the study data: ..... Prof. G. Schlüter
Biometric evaluation: ..... Dr. J. Pauluhn
Head of Department: ..... Prof. Dr. E. Löser
Histopathological evaluation ..... Prof. U. Mohr/Institute of Experimental Pathology[1]
Laboratory Animal Services ..... Dr. K. Hoffmann
Necropsy/macroscopic assessment ..... Dr. M. Rosenbruch
Quality Assurance ..... Dr. H. Lehn
Serological evaluation: ..... Dr. Hildebrand
Study Director and Report Author: ..... Dr. J. Pauluhn
Test substance / stability and purity (HDI): ..... Dr.Diehl
Test substance / stability and purity (TDI): ..... Dr.Pilger
Test substance supply (HDI): ..... Dr.Dislich
Test substance supply (TDI): ..... Dr.Pilger

[1] Insitute of Experimental Pathology, University of Hanover, Germany

7. MATERIALS AND METHODS

7.1. Test Substance

Test substance (I):	**1,6-HEXAMETHYLENE DIISOCYANATE**
Synonymes:	Desmodur H, HDI
Batch-no:	16AX13 HDI-GP / February 2, 1995
Purity:	99.5 % - HDI 0.38 % - Chlorohexylisocyanate 0.34 % Uretdion (dimer)
Producer:	Bayer AG, Leverkusen, Germany
Stability:	guaranteed for the duration of this study and re-confirmed at the end of study. This sample has not been stabilized with Jonol (as it is the case for the commercially available product). Before use the test substance was stripped with N_2.
Appearance:	translucent liquid
Storage:	Room temperature / darkness / under N_2; when not in use the test substance was stored in the refrigerator (ca. 4 °C)
CAS-no.:	822-06-0
Molecular weight:	168.2 g/mol
Molecular formula:	$C_8H_{12}N_2O_2$
Structural formular:	$O=C=N-(CH_2)_6-N=C=O$
Conversion factor:	1 ppm = 6.8 mg/m³ air or 1 mg/m³ air = 0.147 ppm

Test substance (II):	**2,4/2,6-TOLUENE DIISOCYANATE**
Synonymes:	Desmodur T80, TDI
Batch-no:	394
Purity:	≈ 20 % 2,6 - TDI ≈ 80 % 2,4 - TDI The purity has been analytically verified. The purity was 99.98%.
Producer:	Bayer AG, Leverkusen, Germany
Stability:	guaranteed for the duration of this study and re-confirmed at the end of study.
Appearance:	translucent, yellowish liquid
Storage:	Room temperature / darkness / under N_2
CAS-no.:	26471-62-5
Molecular weight:	174.2 g/mol
Molecular formula:	$C_9H_6N_2O_2$
Structural formular:	O=C=N-[CH_3-C_6H_3]-N=C=O
Conversion factor:	1 ppm = 7.2 mg/m³ air or 1 mg/m³ air = 0.14 ppm

Other materials used:

Acetylcholine chloride 98 % (ACh), Aldrich, Cat. No. 13,535-6; vehicle: deionized water

HDI-guinea-pig serum albumin (GPSA): Details concerning the synthesis and characterization of the conjugate are provided in the Appendix of this report (*cf.* Serological Determinations).

TDI-guinea-pig serum albumin (GPSA): so as described for study T1060636 (see 13. APPENDIX II, Conjugate synthesis)

Corn oil, Caesar & Loretz GmbH, batch no. 40079184, dehydrated using molecular sieve Baylith TE 144. The stability of each hapten in the vehicle was confirmed analytically.

7.2. Test system and animal maintenance

Species and rationale: The study was conducted with female guinea-pigs - an animal species recommended for lung sensitization studies.

Young adult, healthy pure-bred guinea-pigs of the DHPW (Dunkin-Hartley Pirbright-White) strain from the Charles River (Crl:(HA)BR, Sulzfeld, Germany were used. This strain of animals has been used for years at Bayer AG for toxicological studies. Historical data on the physiology are available. The state of health of the breed is monitored and the animals are routinely spot-checked for the primary specific pathogens. The results of these tests are retained.

Acclimatization: The animals were acclimatized to the animal room conditions for at least 5 days before use.

Identification: Animals were identified by both individual color-marking and cage-labels. All animals from this study were located on one cage-rack.

Randomization: Before the start of the study the health status of each animal was assessed. Animals were subsequently assigned to exposure groups at random (randomization procedure *vide infra*).

Health status: Only healthy animals free of signs were used for this study. The animals were not vaccinated or treated with anti-infective agents either before their arrival or during the acclimatization or study periods.

Age and weight: At the study start the variation of individual weights did not exceed ± 10 per cent of the mean (see Appendix). Animals of the weight class used are approximately 2 weeks old.

Animal housing: During the acclimatization and study periods four animals per cage were housed under conventional conditions in conventional Makrolon® Type IV cages (based on

A. Spiegel and R. Gönnert, Zschr. Versuchstierkunde, 1, 38 (1961) and G. Meister, Zschr. Versuchstierkunde, 7, 144-153 (1965)). Cages and water bottles were changed twice a week while unconsumed feed was changed once per week. The legal requirements for housing experimental animals (86/609 EEC) were followed.

Bedding: Bedding consisted of type S 8/15 low-dust wood granulate from Ssniff, Soest/Westfalen, Germany. The wood granulate was randomly checked for harmful constituents at the request of the Laboratory Animal Services, Bayer AG.

Animal rooms: All animals were housed in a single animal room in which the following environmental conditions were maintained:

The animal room environment was as follows:

Room temperature:	22 ± 2 °C
Relative humidity:	approximately 50 %
Dark/light cycle:	12 h/12 h; artificial light from 6.00 a.m. to 6.00 p.m. Central European Time
Light intensity:	approximately 14 watt/m² floor area
Ventilation:	approximately 10 air changes per hour

The room humidity and temperature were continuously monitored and documented using a calibrated thermohygrograph. Occasional deviations from these conditions occurred, e.g. as a result of animal room cleaning, but these had no detectable influence on the outcome of this study.

Cleaning, disinfection, and pest control: The animal room was regularly cleaned and disinfected once a week with an aqueous solution of Zephirol®. Contamination of the feed and contact with the test system were excluded. Pest control was not conducted in the animal room.

Feeding: Rations consisted of a standard fixed-formula diet (Altromin® 3022 maintenance diet for Guinea-pigs, Altromin GmbH, Lage) and tap water (drinking bottles). Both feed and

water were available *ad libitum*. The pelletized feed was contained in a rack in the stainless-steel wire cage cover.

The nutritive composition and contaminant content of the standard diet was checked regularly by random sampling by the Laboratory Animal Services, Bayer AG. Details concerning general feed and water specifications are provided in the Appendix.

Water: Drinking quality tap-water (Drinking Water Decree of 05.12.1990, Bundesgesetzblatt [federal law gazette] part I, page 2612) was provided *ad libitum* in polycarbonate bottles containing approximately 700 ml (based on A. Spiegel and R. Gönnert, Zschr. Versuchstierkunde, 1, 38 (1961) and G. Meister, Zschr. Versuchstierkunde, 7, 144-153 (1965)). The results of feed and water analyses are retained by Bayer AG. The available data provided no evidence of an impact on the study objective.

7.3. Test Guidelines

The technical exposure criteria specified in OECD Guideline No. 403 and the corresponding EC Guideline 892/69/EEC (1992) were fulfilled insofar as these are applicable to this study. Other recommendations (US EPA, 1988) were also considered so as to comply with internationally recognized procedures. General recommendations on the techniques used for the for generation and characterization of atmospheres (ASTM E 981-84; Alarie, 1973) and notable recommendations for interpretation (Gross and Vocci, 1988) were observed.

Specific, internationally harmonized test procedures for experiments to assess the lung sensitization potential of low- or high-molecular weight compounds are not currently existing.

7.4. Study design

Sensitization

Recent publications highlight the potential of a simplified test model to examine low-molecular weight agents for their potential to induce lung-sensitization (Pauluhn and Eben, 1991; ECETOC, 1993).

Intradermal induction: Each group consisted of 8 guinea-pigs. For HDI intradermal inductions were performed by three injections on days 0, 2, and 4 using desiccated corn oil as vehicle. For TDI (see Appendix II) animals received a single intradermal induction on day 0. A solution of approximately 0.3% (w/v) was used and an injection volume of 100 µl was given per application. The stability of test substance in desiccated corn oil has been checked analytically on each day of administration. The results can be summarized as follows: HDI: 0.268%, 0.270, and 0.271, and TDI: ca. 0.3%(w/v). There was analytical evidence that the respective concentration of HDI and TDI is stable in the vehicle for at least 6 hours (maximum duration examined).

Combined intradermal and inhalation induction: Additional 8 guinea pigs per group were exposed by inhalation on five consecutive days, 3 hrs per day, target concentration 40 mg/m³ air. Due to the marked respiratory signs observed in the HDI-exposure group following the first exposure day the target concentration of 40 mg/m³ air was reduced to 20 mg/m³ air (day 1 - 4). One intradermal injection was given on day 0 under conditions described above.

Challenge

Group allocations/schedule for challenge: Challenge was conducted following a recovery period of about two to three weeks after sensitization. Details are provided in the Appendix.

Group allocations/schedule for challenge

Group	AN	Induction		Elicitation	
		Substance	Regime	Day[1]	Aerosol[2]
1	1-8	Vehicle control (id)	3 x 100 µl (day 0, 2, 4)	21 / 22 / 23 / 28	ACh/HDI/ACh/Conj
2	9-16	HDI (id)	3 x 100 µl (day 0, 2, 4)	21 / 22 / 23 / 28	ACh/HDI/ACh/Conj
3	17-24	HDI (id / ih)	1 x 100 µl(day 0) 5x3h (day 0-4)	21 / 22 / 23 / 28	ACh/HDI/ACh/Conj
4	25-32	TDI (id / ih)	1 x 100 µl(day 0) 5x3h (day 0-4)	21 / 22 / 23 / 28	ACh/TDI/ACh/Conj
1'	1-8	Vehicle control (id)	1 x 100 µl (day 0)	21 / 22 / 23 / 28	ACh/TDI/ACh/Conj
2'	9-16	TDI (id)	1 x 100 µl (day 0)	22 / 23 / 24 / 29	ACh/TDI/ACh/Conj

AN = Guinea pig number (due to software reasons the animal nos. presented in the Tables/Figures in the Appendices may not necessarily be ordered in this sequence).
Vehicle = Dehydrated corn oil
Regime = 1 x: single intradermal injection (day 0) using 100 µl/injection;
3 x: triple intradermal injections, one each day using 100 µl/injection
1) = Day of challenge specified in 2)
ACh = Ramped exposure to 0.1, 0.2, 0.4 and 0.8 % concentrations of ACh sequentially for 15 minutes each.
1', 2' = Separate study (T3060700), for individual results see 13. Appendix II

All animals were challenged using an identical protocol and with almost identical target concentrations of HDI, TDI and ACh.

7.5. Exposure Conditions

Mode of exposure: Animals were exposed to the evaporated test substance in Plexiglas exposure tubes applying a *directed-flow* nose-only exposure principle. Tubes were chosen that accommodated the animals size. This type of exposure is preferable to whole-body exposure on scientific (Pauluhn, 1994a) and technical reasons (rapid attainment of steady-state concentrations, no problems with regard to test atmosphere inhomogeneities, better capabilities to control all inhalation chamber parameters, easier cleaning of exhaust air, easily

accessable plethsymographic technique). Moreover, contamination of the fur can largely be avoided. The chambers used are commercially available (TSE, 61348 Bad Homburg) and the performance of this type of chamber has been published (Pauluhn, 1994a).

Vehicle: HDI and TDI test atmospheres were generated as vapor without using an additional carrier or vehicle.

7.6. Generation of Atmosphere and Exposure Technique

7.6.1. Generation of HDI/TDI-Atmospheres

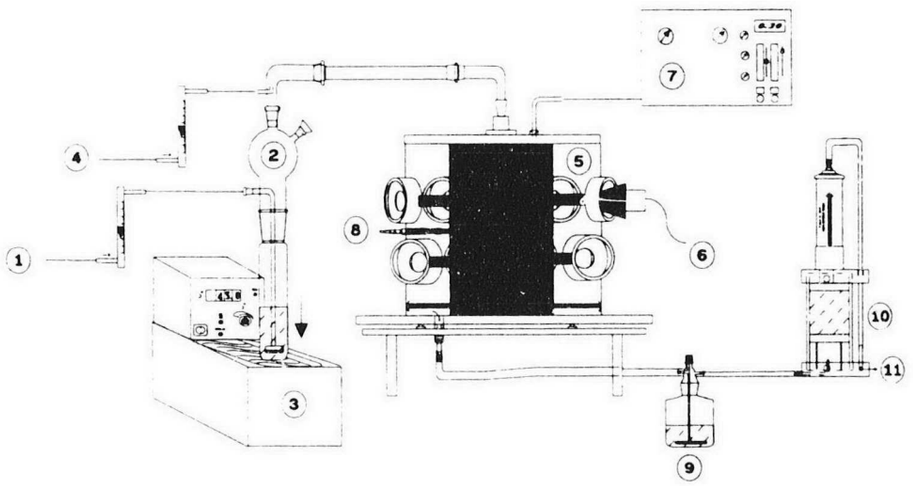
Under dynamic conditions the test substance was fed into the intake of the cylindrical inhalation chamber so as shown in Figure 1. Dry conditioned air or nitrogen (flow rates see Table 1 in the result section) was fed through the liquid of the test substance contained in a glass bubbler (diameter: ≈ 5 cm, hight of liquid level: ≈ 5 cm, content ca. 90 ml) using a calibrated flow meter and was subsequently diluted with conditioned dry air to achieve the target concentration (*cf.* Table 1 in the result section). The glass bubbler containing the test compound was maintained at 45 °C using a thermostate (JULABO UC, Julabo, Seelbach, Germany). For TDI two parallel glas bubblers, each supplied with 0.9 l air/min, were used. For challenge exposures smaller glas bubblers (content ca. 8 ml) were used.

The *directed-flow* arrangement of this type of nose-only inhalation chamber minimizes re-breathing of exhaled test atmosphere. Also the degradation/hydrolysis of the test atmosphere as a result of contact with humidified exhaled air is minimized or even impossible due to the design of the inhalation chamber. The stability of the test atmosphere was monitored continuously using a total hydrocarbon analyzer equipped with a flame ionization detector (Compur, Munich, Germany). The inhalation chamber used consisted of one segment suitable to accommodate 20 rats at the perimeter location. All air flows are monitored and adjusted continuously by means of flow-controllers. A soap bubble meter (Gilibrator, Ströhlein Instruments, Kaarst, Germany) was used to monitor the accuracy of flow-controllers. As demonstrated in Table 1, the ratio between main supply and exhaust air was selected so that ca. 80% of the supplied air was extracted via the exhaust air location and, if applicable, via sampling ports. Activated charcoal was used for exhaust air clean-up. The slight positive balance between the air volume supplied and extracted ensured that no passive influx of air into the exposure chamber occurred (via apertures). The remainder provides also adequate

dead-space ventilation of the exposure tubes. The pressure difference between the inner inhalation chamber and the exposure zone was 0.02 cm H_2O (Pauluhn, 1994a). The exposure system was accommodated in an adequately ventilated enclosure.

Inhalation Chamber: The aluminum inhalation chamber has the following dimensions: inner diameter = 14 cm, outer diameter = 35 cm (two-chamber system), height = 25 cm (internal volume = about 3.8 l). The construction of the inhalation chamber is shown schematically in Fig. 1. Details of this modular chamber and its validation with regard to spatial homogeneity of material distribution have been published (Pauluhn, 1994a).

Fig. 1: Inhalation Chamber (schematic)



1. Air supply	7. Real-time monitoring (THC)
2. Test substance in glass bubbler	8. Sampling location ('breathing zone sampling')
3. JULABO thermostate	9-10. Make-up of exhaust air, including HEPA-filter / activated char choal
4. Dilution air	11. Exhaust air
5. Exposure zone	
6. Sensor for temperature and humidity measurement	

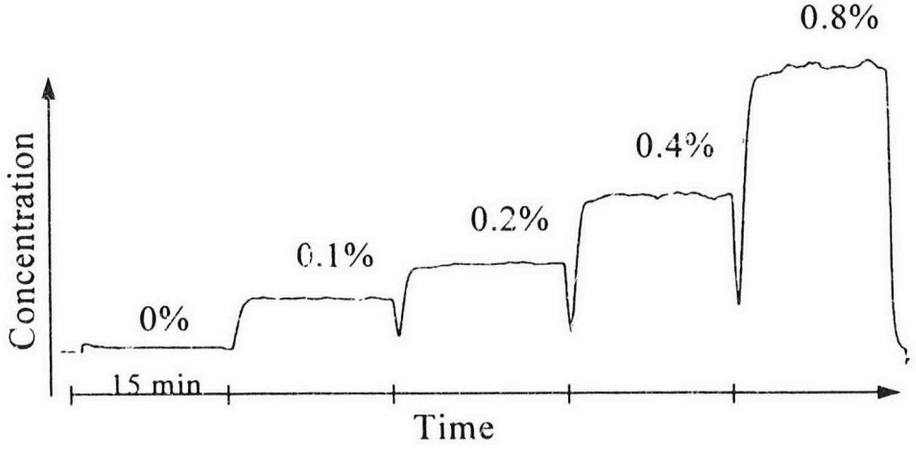
Inhalation chamber steady-state concentration: The test atmosphere generation conditions provide an adequate number of air exchanges per hour (> 300 x, continuous generation of test

atmosphere). Under such test conditions steady state is attained within the first minute of exposure ($t_{99\%}$ = 4.6 x chamber volume/flow rate; McFarland, 1976). As alluded to earlier, the ratio between the air supplied and exhausted was chosen so that approximately 80-90% of the supplied air is removed as exhaust. The remainder provides adequate dead-space ventilation for the exposure tubes. At each exposure port a minimal air flow rate of 1.4 l/min was provided. The test atmosphere can by no means be diluted by bias-air-flows. The inhalation chamber was operated in a well ventilated chemical fume hood.

7.6.2. Generation of Acetylcholine Aerosol -Atmospheres

ACh was dissolved in deionized water to obtain concentrations of 0.1, 0.2, 0.4, and 0.8 % (w/v), and these solutions were nebulized into the baffle at a rate of 75 µl per minute. Increasing concentrations were achieved by the nebulization of subsequently increased spray solutions. The attained aerosol increased proportionally with increased concentration of the nebulized solution (Fig. 2).

Fig. 2: Ramped ACh-Challenge - Real-time monitoring



A binary nozzle was used and 15 liters of air per minute was supplied at a dispersion pressure of approximately 400 kPa. The spray solution was continuously fed to the nozzle using a Braun® infusion pump. Before entering the inhalation chamber larger particles were

eliminated from by a baffle/seperator. During ACh-challenge the inhalation chamber temperature was ca. 21 °C, the respective relative humidity was ca. 30%. Further details are depicted in Fig. 3.

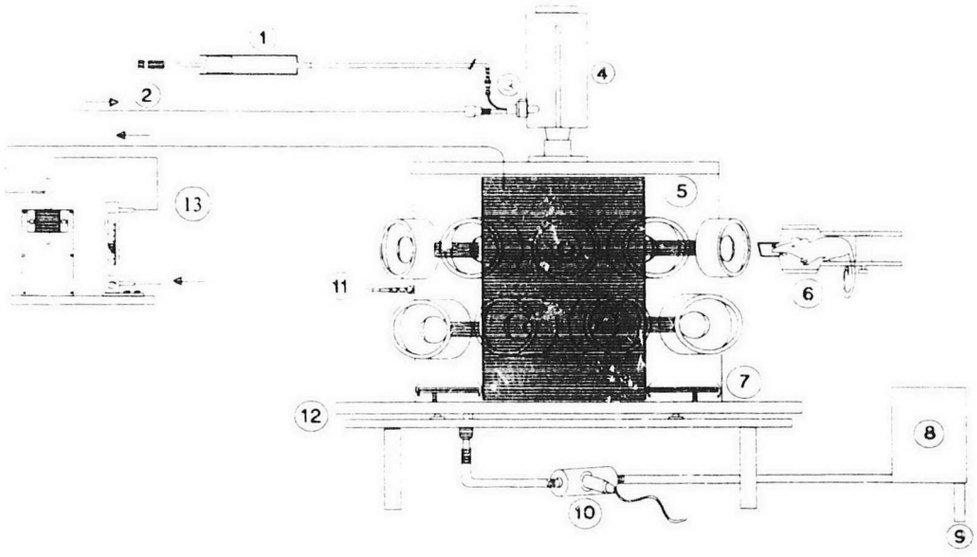
7.6.3. Generation of Conjugate Aerosol -Atmospheres

The respective hapten GPSA-conjugate (for characterization see Appendix) was dissolved in saline to obtain a concentration of 1.5 % (w/v), and this solution was nebulized into the baffle at a rate of 200 µl per minute. Again a binary nozzle was used and 10 liters of air per minute was supplied at a dispersion pressure of approximately 200 kPa. The spray solution was continuously fed to the nozzle using the system so as shown in Fig. 3. During conjugate challenges the inhalation chamber temperature was ca. 21 °C, the respective relative humidity was ca. 40-50%.

Respirability optimization: For liquid aerosols the preseparator/baffle system was used to increase the aerosolization efficiency and to prevent larger particles from entering the inhalation chamber (Tillery et al., 1976). Details of this aerosol generation system have been published elsewhere (Pauluhn, 1994a). The dimensions of the baffle section were 10 x 10 x 19.5 cm (length x width x height).

Air flows: During the exposure period, air flows were continuously monitored and readjusted to nominal settings as required. Generally, air flows were measured using calibrated flowmeters. These calibrated flowmeters were checked with bubble-meters (Gilibrator) for proper performance prior to the study and at regular intervals during the study.

Fig. 3: Inhalation chamber - Conjugate Aerosol Challenges



1. Test substance supply	8. Cotton-wool/activated charcoal aerosol filter (air make-up)
2. Compressed air	9. Flow meter to monitor exhaust air
3. Nozzle	10. Sensor for temperature and humidity measurement (actual location: exposure port)
4. Baffle	11. Sampling location ('breathing zone sampling')
5. Inhalation chamber	12. Rotatable base
6. Plethysmograph	13. Photometer (Real-time monitoring)
7. Air outlet (exhaust air)	

Compressed air conditioning: The compressed air was produced with Boge compressors. The air was automatically conditioned (i.e. water, dust and oil removed) by subsequent passage through a VIA compressed air dryer. The regulated operating pressure of the compressors was 8 - 10 bars (800 - 1000 kPa). Pressure-reduction valves were used to set the operating pressure.

Treatment of exhaust air: The exhaust air was purified through cotton-wool/activated charcoal and HEPA filters. These filters were disposed of by Bayer AG.

7.7. Inhalation Chamber Temperature and Humidity

Temperature and humidity values were determined using the Leybold-Heraeus system as described below. Readings were recorded at 10-minute intervals. Throughout all exposures the sensor was located in the exhaust location of the inhalation chamber. The humidity-detecting cell was protected against aerosols by a Teflon® membrane (pore size about 1 µm) sandwiched between two sintered-metal filters. The humidity sensors were calibrated with saturated salts solutions (Greenspan, 1977; Pauluhn, 1986). The temperature sensors were calibrated with standard thermometers. Readings were transmitted through an IEEE 488 interface and recorded and analyzed using an Apple IIe computer equipped with an MDP 8240/45 analog/digital converter. During the conjugate challenge humidity was monitored using a Lambrecht hygrometer (location of sensor: exhaust air) and digital thermometer (location of sensor: breathing zone area). During the induction period a Lambrecht Hygrometer was used.

7.8. Analysis of the Test Atmosphere

7.8. 1. Analysis of HDI/TDI Test Atmospheres

The nominal concentration was calculated taking into account the actually evaporated mass of test substance (difference of weight of the glass bubbler before and after exposure) devided by total airflow through the chamber. The mass loss during the challenge was too low to allow the calculation of nominal concentrations.

The test atmosphere was determined by HPLC after derivatization of the isocyanate functionality. Samples were taken by using glass powder filled tubes containing nitroreagent as scavenging agent. Further methodological details related to sampling as well as characterization of test atmosphere are provided in the Appendix.

Chamber samples were taken in the vicinity of the breathing zone (see Fig. 1). The number of samples taken was sufficient to characterize the test atmosphere and was adjusted so as to accommodate the sampling duration and/or the need to confirm specific concentration values. Optimally, samples were collected after the equilibrium concentration had been attained in hourly intervals. All analytical concentrations reported refer to mg of test substance/m³ air.

7.8.2. Analysis of Acetylcholine chloride Test Atmospheres

The ACh aerosol was indirectly quantified from samples taken in the breathing zone area using a TSI laser velocimeter (see below).

7.8.3. Analysis of Conjugate Test Atmospheres

For gravimetric determinations of the hapten-GPSA conjugate glass fiber filters were used (SM 13430, Sartorius, Göttingen, Germany). Filter weights were determined using an electronic balance (Mettler AE 100, Göttingen, Germany). The flow rate during sampling was 4 liter/minute and the volume was approximately 50 liters of air per sample in total.

7.9. Stability of Test Atmosphere

The stability of the aerosol generation system(s) was checked using a RAM-1 or RAS-2 aerosol photometer (MIE, Bedford, Massachusetts, USA). The integrity and stability of the vapor generation system was checked continuously using a Compur Total Hydrocarbon Analyzer (equipped with FID) (Compur, Munich, Germany).

Samples were taken continuously from the vicinity of the breathing zone. This chamber monitoring allows for an overall survey of toxicologically relevant technical parameters (inlet and exhaust flows as well as atmosphere homogeneity, temporal stability, and generation performance). Interruptions in exposure (e.g. resulting from obstruction of the nozzle or other technical mishaps) were recorded and, if applicable, a commensurate interval was added to the exposure duration for compensation.

7.10. Test atmosphere particle characterization

7.10.1. Evaluation of particle-size distributions / Acetylcholinprovocation

Samples for the analysis of the aerodynamic particle-size distribution were also taken in the vicinity of the breathing zone. These samples were taken using a TSI-Laser Velocimeter APS 3300, including diluter TSI Model 3302 (TSI Inc., St. Paul, MN, USA). Technical details of this system have been described (Remiarz et al., 1983). The TSI-Laser Velocimeter APS is checked and calibrated at regular intervals by TSI (TSI, 1986). Particle size measurements were conducted once during the experiment. Representative examples are provided in the Appendix.

For the APS 3300/diluter equipment the cumulative number distribution is used to calculate the number median aerodynamic diameter (NMAD) and geometric standard deviation (GSD). The NMAD and GSD are determined from the probit-transformed cumulative particle number frequency distribution (y-axis) and the logarithmic effective cut-off diameters (ECD's) (x-axis) of the individual channels by linear regression. The GSD is calculated from the regression line: percentile 84 / percentile 50. The MMAD is then calculated from the NMAD using the following formula (Raabe, 1970; Marple and Rubow, 1980; Pauluhn, 1994a).

$$MMAD = NMAD \times \exp(3 \ln^2 GSD)$$

7.10.2. Evaluation of particle-size distributions / Conjugate

Representative samples for analysis of particle-size distribution were taken in the vicinity of the breathing zone area during the induction exposures and before or after challenge. The sample was taken using a low-pressure cascade impactor. Specifications and evaluations are provided in the Appendix. The individual impactor stages were covered with aluminum foil and a glass fibre filter and were evaluated by gravimetric analysis. Silicon spray was not used for adhesive coating for the aluminum foil surfaces to prevent particle bounce because of the presence of the filter.

For the evaluation of the cascade impactor analyses the mass median aerodynamic diameter (MMAD) and the geometric standard deviation (GSD) are determined from the probit-transformed cumulative particle mass frequency distribution (y-axis) and the logarithmic effective cut-off diameters (ECD's) (x-axis) of the individual impactor stages by linear regression. The GSD is calculated from the regression line: percentile 84 / percentile 50. The relative mass with an aerodynamic diameter ≤ 3 µm ("respirable mass fraction") [Raabe, 1982; Snipes, 1989; SOT-Commentary, 1992] is calculated from the regression line. For probit transformation and linear regression FORTRAN algorithms are used.

To verify whether the aerosol distribution is in fact unimodal and log-normal the normalized mass per stage (f_H') is evaluated as a histogram. $\Delta \log D_p$ is equal the difference $\log D_{p+1}$ - $\log D_p$, whereas D_p is the lower (left) cut-size limit and D_{p+1} the higher (right) cut-size limit of the corresponding impactor stage. As demonstrated by the evaluations included in the Appendix, the impactor stage cut-off limit (D_{p+1}) to the right was used for all calculations.

$$f_H' = \frac{1}{N_f} \times \frac{mass \ / \ stage}{\Delta \log D_p}$$

The log-normal mass distribution $y'(D_{ae}) = 1/N_f \times y(D_{ae})$ as a function of the aerodynamic diameter (D_{ae}) is computed using the formula:

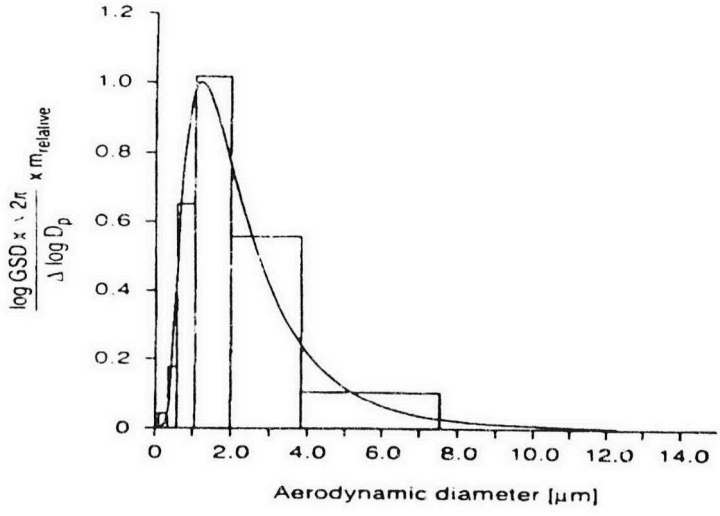
$$y'(D_{ae}) = \exp\left[-\frac{(\log D_{ae} - \log MMAD)^2}{2 \times \log^2 GSD} \right]$$

The normalization factor (N_f) is calculated as follows:

$$N_f = \frac{\Sigma mass}{\log GSD \times \sqrt{2\pi}}$$

Where Σmass is the total mass collected by the cascade impactor, where $m_{relative}$ = mass per stage/Σmass (*cf.* Fig. 4).

Figure 4: Principle of characterization of aerosol atmosphere



The algorithm for the calculation of particle size characteristics is taken from pertinent reference works on aerosol physics (Dennis, 1976; Marple and Rubow, 1980) and proves to be generally applicable (Pauluhn, 1994a).

Respirability

The particle-size distribution achieved is adequately respirable to reach all potential targets within the guinea-pigs' respiratory tract (Snipes, 1989).

7.11. Collection efficiency

The sampling equipment was adjusted with calibrated rotameters to internationally recognized standards (ACGIH, 1978; Section I "Calibration of Air Sampling Instruments").

The conditions for test atmosphere generation were optimized to provide maximum aerosol respirability to laboratory animals (Raabe, 1982; Snipes, 1989; SOT-Commentary, 1992).

The absence of larger particles and high flow rates in the vicinity of the sampling ports make it possible to disregard potential anisokinetic sampling errors, thus ensuring a representative sampling even with different sampling probe orifice diameters and flow rates. The tolerance limits for the radius of the probe orifice are calculated using the following formula [ACGIH, 1978]. Calculations consider both a particle-size distribution that encompasses aerodynamic diameters (D_{ae}) of 0.5 to 7.4 µm and sample flows ranging from 8 to 80 ml/sec.

$$5 \times \sqrt[3]{\frac{flow \times \tau}{4 \times \pi}} \leq r_p \leq \frac{1}{5} \times \sqrt[2]{\frac{flow}{g \times \tau \times \pi}}$$

r_p = radius of the sample probe in cm = ½ x D_p
τ = relaxation time (D_{ae} 0.5 µm = 1×10^{-6} sec; D_{ae} 7.4 µm = 1.7×10^{-4} sec)
g = gravity constant = 980 cm/sec^2

Tolerance limits calculations for the sample probe orifice (r_p) indicated that a representative sampling is assured when the orifice inner diameter is in the range of 1.0 to 1.6 cm. Orifices of the sampling instruments used here are consistent with this criteria. Details of the D_p tolerance limit calculations are published elsewhere (Pauluhn, 1994a).

7.12. Body weights

The body weights were determined prior to induction, on relative study days three and seven, and weekly thereafter. Animals were also weighed before necropsy.

7.13. Clinical signs

If applicable, the appearance and behavior of each guinea-pig was examined carefully at least twice per day of exposure and at least once daily thereafter (including weekends). Assessments from restraining tubes were made only if unequivocal signs occurred (e.g. spasms, abnormal movements, severe respiratory signs). Following exposure, observations are made and recorded systematically; individual records are maintained for each animal.

Cageside observations included, but were not limited to, changes in the skin and fur, eyes, mucous membranes, respiratory, circulatory, autonomic and central nervous system, and somatomotor activity and behavior pattern. Particular attention was directed to observation of tremors, convulsions, salivation, diarrhea, lethargy, somnolence and prostration.

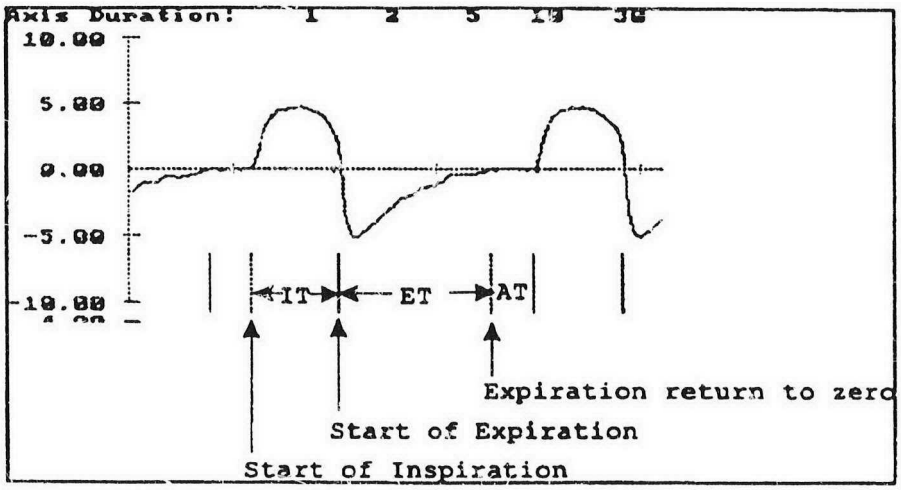
7.14. Immediate-onset lung function measurements

Immediate-onset reactions: Measurements were conducted with spontaneously breathing, conscious guinea pigs in modified nose-only exposure tubes used as plethysmographs. The animals were acclimatized to the exposure conditions for an adequate period of time. Animals were considered acclimatized when the respiratory rate reached roughly 90 breaths per minute.

After acclimatization baseline parameters were measured for approximately 15 min (exposure to air). The duration of exposure to the test substance was approximately 30 min, followed by post-challenge measurements of approximately 60 minutes (for a detailed itemization of responses *cf.* Appendix/Lung Function Measurements). Measurements were made with eight animals simultaneously. For evaluation of responses occurring during challenge exposures the following respiratory parameters were evaluated: respiratory rate (RR) [breaths/min], tidal volume (TV) [ml], respiratory minute volume (MV) [ml/min], peak inspiratory and expiratory flow rates (PIF and PEF) [ml/sec], inspiratory (IT) and expiratory times (ET) [msec], the average duration of apnoic period (AT) [msec], and the number of apnoic periods per logging period exceeding 20% of the ET period [incidence/logging period]. Additional parameters were derived as shown in the Appendix. Measurements were made in nose-only animal restrainers with wire-mesh style pneumotachograph and differential pressure transducers (MP 45 ± 2 cm H_2O, Validyne) fitted shortly onto the plethysmograph. The head and body compartments were separated using a double-layer latex neck seal. Precautions were taken to avoid artifacts due to restraint and tight fitting seals around the neck. Volumes were calculated by integration of the flow signal from the body compartment and potential artifacts related to the dependence of the calculated volume as a function of respiratory frequency were considered (Pauluhn, 1994b). The resistance to air flow of the wire-mesh screens was

adjusted so that artificial volume changes between pump rates of 50-250 cycles/min did not exceed 10%. The validation of the system was performed prior to each exposure individually for all plethysmographs using a calibration volume of 2.0 ml at a frequency of 150 cycles/min. In most instances, the signals were averaged during a logging period of 20 seconds. The flow and volume signals for each individual animal were displayed on the monitor of the PC during measurement. Phase and amplitude checks were documented by re-processing of raw data. The principle of the evaluation of breathing patterns is illustrated in the following Figure 5.

Figure 5: Flow/volume measurements



Data recording and evaluation: Individual baseline data were used to calculate the mean ± 3 and 4 x standard deviation (STD). Responses exceeding the mean ± 3 x STD were considered to be positive. A rank order of responses was made. The highest rank was given to increased values of the respiratory rate (hapten and conjugate challenge) and the PEF x (ET+IT)/TV parameter. Data provided in the graphs presented in the Appendix were smoothed by a low pass filter to eliminate high frequent breathing patterns.

Analyses were additionally perfomed 'on-line' on non-smoothed data. This evaluation of data

counted the number of events (each averaged period of 20 sec) above the individual mean + 3 x STD. To allow comparison with pooled control data, the respective pre-exposure control period (mean + 1 STD) was evaluated to calculate the prediction intervals for the breathing parameters of interest.

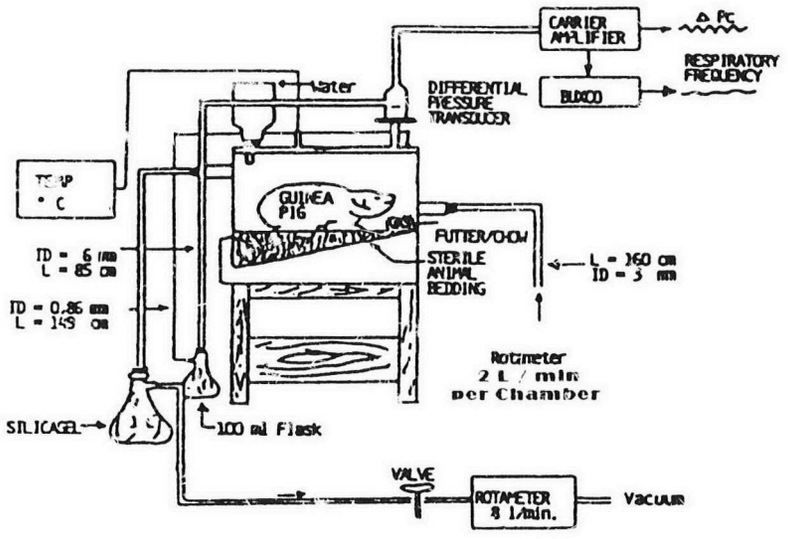
Acetylcholine provocation: Results of the acetylcholine bronchoprovocation assay were evaluated using an iterative mathematical approach (formula see below). For evaluation of the concentration-response curve of controls all pooled controls were used. For TDI (due to the limited number of data available) the two parameters fitted to the curve were the parameters p_1 (amplitude) and p_3 (slope).

$$y = p_1 / (1 + \exp(p_2 \times (x - p_3)))$$

7.15. Delayed-onset lung function measurements

Four animals per group of study T3060700 (*cf.* 13. Appendix II) were subjected to measurements for delayed-onset responses. A delayed-onset respiratory hypersensitivity response was indicated by an increased RR as described by Karol *et al.* (1985), Karol and Thorne (1988). This endpoint was evaluated from unrestrained guinea-pigs using water-jacketed whole-body plethysmographs (temperature approximately 21.5 ± 1 °C, duration of measurement: ca. 20 hours; bias air-flow rate 2 l/min) (laboratory thermostat; Julabo UC - 5B/5). The volume of the bias-flow whole body plethysmograph was 2.44 liter (length = 23.5 cm, width = 11.5 cm). The comparison of RR values in unrestrained (whole-body bias flow plethysmographs) and restrained guinea-pigs (nose-only plethysmographs) revealed a baseline RR value of approximately 90 breaths/min for both systems. Thus animals were apparently not unduly stressed during restraint. Details of this system have been published previously (Pauluhn and Eben, 1991). Respiratory rate data were reported as one minute integrations and results were averaged over five-minute intervals for tables and Figures. The test principle is illustrated in the following Figure 6.

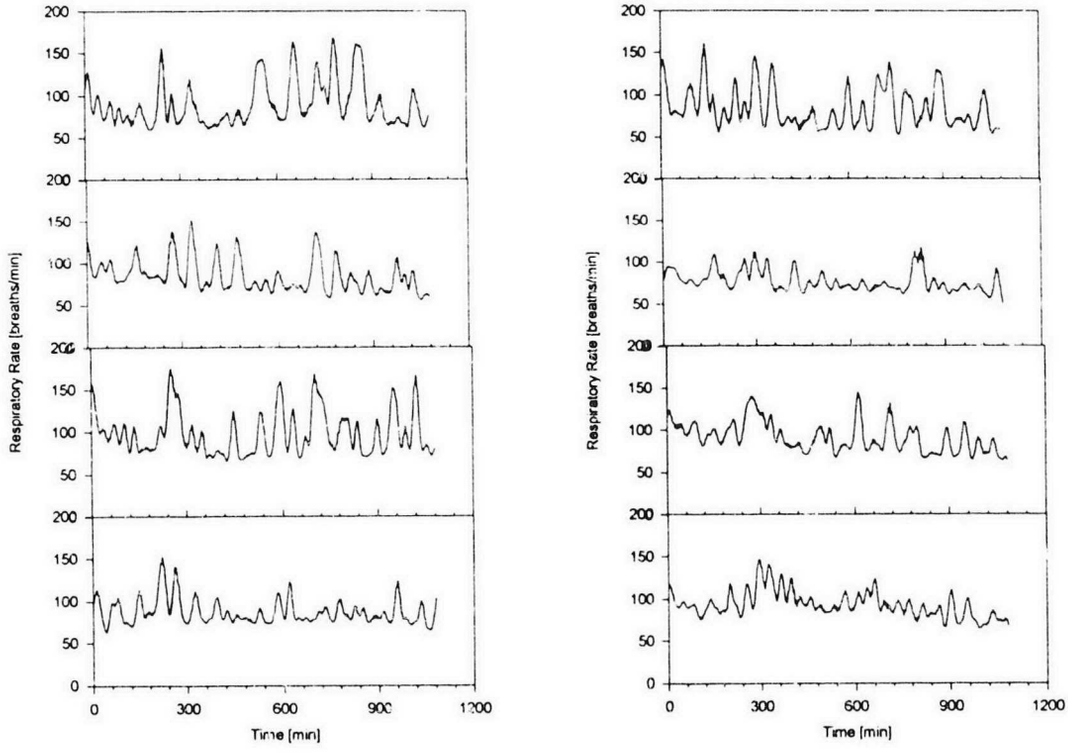
Figure 6: Schematic for measurement of delayed-onset reactions.



Evaluation: Previous analysis of the respiratory rate (RR) over a period of about 20 hours yielded a mean RR of approximately 90 and a single SD of ca. 20 (Pauluhn and Eben, 1991). Accordingly, a temporary rise in the respiration rate to 90 + 2 SD was taken as a positive reaction, and an increase to 90 + 3 SD as a strongly positive reaction.

Examples of this type of evaluation are summarized in Fig. 7. However, it must be emphasized that, due to changes observed also in controls, the bias-flow plethysmography is apparently not the methodology of choice to examine small changes in brathing pattern.

Figure 7: Results of delayed-onset measurements. Panel left: Controls after TDI-challenge, panel right: single intradermal induction after TDI-challenge



7.16. Necropsy and Histopathology

Necropsy. Animals were sacrificed one day after the final challenge. Intraperitoneal injection of sodium pentobarbital (approx. 600 mg/kg b.w.) was used for euthanasia. The animals were then examined for gross pathologic changes. All findings deviating from normal were documented. Complete exsanguination was performed through cardiac puncture and the blood collected was used serological determinations. Following exsanguination the lung weights were determined.

Histopathology. The lung, trachea, and lung associated lymph nodes were subjected to histopathological evaluation with particular emphasis to eosinophil infiltration. Further methodological details are provided in the respective Appendix.

7.17. Serological Determinations

At termination, several milliliters of blood were collected from each animal and was allowed to clot at room temperature for approximately one hour. The samples were then stored overnight at ca. 4 °C to complete the clotting process. After centrifugation, serum was collected and stored at -20 °C prior to consigning to Dr. Hildebrand. Details concerning the preparation of the conjugate, its characterization, the methodology, and results of serological determinations are reported in the respective Appendix.

7.18. Statistical evaluation

Body weights: Body weight gains were analyzed by *one-way* analysis of variance and Tukey-Kramer *post hoc* test (BCTIC Computer Code Collection - Biomedical Computing Technology Information Center. ANOVA a FORTRAN Program to Perform one-way Classification Analysis of Variance. Vanderbilt Medical Center, Nashville, Tennessee, USA). The criterion for statistical significance was set at $p < 0.05$.

Lung weights: Lung weights (absolute and relative *versus* body weights) were analyzed by *one-way* analysis of variance and Tukey-Kramer *post hoc* test (BCTIC Computer Code Collection - Biomedical Computing Technology Information Center: ANOVA a FORTRAN Program to Perform one-way Classification Analysis of Variance. Vanderbilt Medical Center, Nashville, Tennessee, USA). The criterion for statistical significance was set at $p < 0.05$.

Pulmonary function tests: Absolute and relative values for each parameter are reproduced in tabular or graphical form in the Appendix. All parameters collected are also reproduced graphically and these data were smoothed using a lowpass filter before graphing (low pass

filter for high frequency outliers). Brief peaks caused by abnormal movements in the plethysmograph were thereby minimized. Data in tables reflect the raw data.

One-way analysis of variances (ANOVA): In this parametric method, the data are checked for normal distribution by comparison of the median and mean values. The variances between the groups were tested for homogeneity with Box's test. If the F-test showed that the variation within the group was greater than that between the groups, this fact is indicated in the Appendix by the remark "no statistical difference between the groups". If a difference was determined, a pairwise *post-hoc* (one and two-tailed) comparison of the groups was performed using the Games and Howell modification of the *Tukey-Kramer* significance test.

Histopathology findings: If specific findings occur from the respiratory tract of surviving rats they are evaluated statistically using the pairwise Fisher test after the R x C chi-squared test (HP 3000, Department of Toxicology, Bayer AG). The Fisher test was only performed if differences occurred between groups in the R x C chi-squared test or if a frequency value of < 5 was calculated. This procedure was performed in accordance with Gad and Weil (1982). For calculation of the unilateral p value a symmetrical distribution was assumed (p unilateral = (p bilateral)/2).

Randomization: The randomization lists were produced with the aid of a computer program which used a random number generator.

7.19. Reproduction of Raw Data

Raw data entered into, processed by and/or stored in a computer system could be saved and printed out in various formats. The precision (number of decimal places) of the values printed and reproduced in this report reflect toxicologically relevant levels of precision. Deviations between manually calculated and computer-determined values can arise due to rounding. Values with no decimal places do not necessarily represent the pertinent measurement precision of the detection system.

7.20. Software Programming and Validation

Software code for the following purposes was written in HP Fortran (HP 3000) or Microsoft Fortran 77 (PC): particle-size analysis, ANOVA, Fisher test, inhalation chamber data tabulation program, graphics software, physiological data evaluation. All scratch files were generated using Fortran F8.3 format using the Fortran default rounding routines. Fortran format A was always used to generate alphanumeric tables and graphs; i.e. numbers in figures and tables are rounded-up or -off due to the different format codes of the server. The computer programs were carefully validated. The validation was conducted using text book data sets (Gad and Weil, 1982). However, it should be taken into account that the formal requirements of the GLP-principles for validation of computer software are not fulfilled. Wherever possible, raw data and calculated values are displayed graphically to provide a versatile opportunity for data comparison.

7.21. Raw Data and Report Archival

The protocol, raw data, specimens, and the final report are archived in locations specified by Bayer AG, in accordance with GLP requirements.

8. RESULTS

8.1. Induction of Animals

It could be demonstrated that for intradermal inductions the target concentrations of HDI and TDI of approximately 0.3% were met. There was analytical evidence that the respective concentration of HDI and TDI is stable in the vehicle (desiccated corn oil) on the day of administration.

Technical information concerning generation of test atmospheres is provided in Table 1a.

Table 1a: Characterization of induction atmospheres

	Group 3	Group 4
Induction Group	**HDI**	**TDI**
Mean Actual Conc. (mg/m³)	27.4 ± 7.1	51.4 ± 2.6
Inlet Air Flow (l/min)	20	20
Total exhaust air flow (l/min)	18	18
Temperature (mean, °C)	22	17
Rel. Humidity (mean, %)	22	18

Analytical as well as real-time monitoring of each test atmosphere indicated that the exposure conditions were temporally stable over the induction period. The 5 x 3 hrs inhalation exposures (ih) to the respective vapor atmosphere resulted in guinea pigs exposed to HDI more pronounced signs of respiratory tract irritation when compared with TDI. Therefore, after the first exposure day, the target concentration of HDI was reduced by 50% (see Appendix).

Temperature values in the inhalation chamber were in the range suggested by the testing guidelines. Humidity values were lower, this is undoubtedly related to the use of dry air for generation. However, this deviation from the OECD guideline has no negative impact on the study results.

The results obtained during and after the various induction encounters to HDI or TDI are summarized in Table 1b.

Table 1b: Summary of acute inhalation toxicity - Induction period

Group	Regimen	Toxicological Result[1]	Onset and Duration of Signs[2]	Onset of Mortality (%)
1	vehicle	0 / 0 / 8	--	--
2	id - HDI	0 / 0 / 8	--	--
3	id + ih - HDI	0 / 8 / 8	0d - 6d	--
4	id + ih - TDI	0 / 8 / 8	0d - 6d	--
1'	vehicle	0 / 0 / 8	--	--
2'	id - TDI	0 / 0 / 8	--	--

1) Duration of signs related to respiratory tract irritation only (for reactions at injection site see Appendix).
2) Day relative to the first induction day (= day 0)

Values given in the 'Toxicological results' column are:
1st = number of dead animals.
2nd = number of animals with signs after cessation of exposure.
3rd = number of animals exposed.

Signs and observations:
All signs are tabulated in the Appendix in incidence tables.

Groups 1 and 1': All guinea-pigs tolerated the treatment without specific signs.

Groups 2: Injection site reddened, dark discolouration, oedematous, serous exsudation, necrotic, transient reddening of skin not covered by hair-coat.

Groups 2': Injection site reddened, dark discolouration, oedematous, serous exsudation, necrotic, transient reddening of skin not covered by hair-coat.

Group 3: Injection site reddened, dark discolouration, oedematous, necrotic, transient reddening of skin not covered by hair-coat. Rales, laboured breathing pattern, bradypnea, cyanotic appearance, cough like breathing sounds, dyspnea, serous discharge from nose.

Group 4: Injection site reddened, dark discolouration, necrotic, transient reddening of skin not covered by hair-coat. Rales, laboured breathing pattern, bradypnea, cough-like breathing sounds, serous discharge from nose.

The intensitiy of signs was more pronounced in group 3 when compared to groups 4.

Following intradermal induction (id) most animals showed marked inflammatory responses at the injection sites which persisted throughout the observation period (for details see Appendix).

Body weights: Animals induced by inhalation experienced a mild and transient decrease in body weight gains relative to the respective vehicle control. All body weight data, including their statistical analysis, are reproduced in the Appendix.

Lung weights: Marked differences in lung weights between the group were not evident in the groups. However, it appeared that the lung weights of animals induced by TDI showed a tendency of elevated lung weights. All data and the statistical analysis of this data are reproduced in the Appendix.

Gross pathological examinations: Equivalent findings were observed from the vehicle and the induction groups (see Appendix).

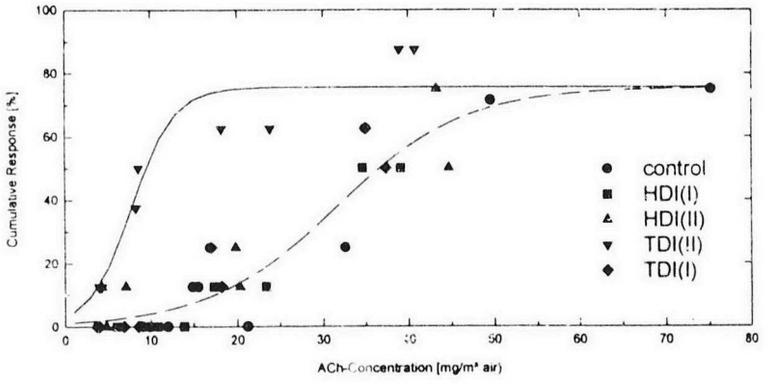
8.2. Elicitation of Respiratory Hypersensitivity by Hapten an ACh-Challenge

The results of challenge exposures with the haptens HDI or TDI can be summarized as follows: Guinea pigs challenged with average hapten concentrations of approximately 0.5 mg/m³ did not experience any conclusive change in breathing pattern. The results of the acetylcholine bronchoprovocation assay performed one day before and one day after the respective hapten challenge were almost identical, thus indicating that differences in hyper-responsiveness are apparently related to induction inhalation exposures rather than hapten

challenge.

Acetylcholine provocation: The highest rank was given to increased values of the derived dimensionless parameter 'PEF x (ET+IT)/TV'. The results of the acetylcholine broncho-provocation assay is evaluated using an iterative mathematical approach. For evaluation of the concentration-response curve of pooled controls were used. As depicted in Fig. 8, guinea pigs subjected to repeated TDI inhalation exposures (group 4) experienced a conspicuous non-specific airway hyperreactivity. This demonstrates that previous encounters by inhalation with high concentrations of the inciting agent TDI rendered the animals hyperreactive to non-specific stimuli. The effective concentration provoking 50% response (EC_{50}) was in non-hyperreactive guinea pigs approximately 32 mg ACh/m³ air (p_1=75.5, p_2=0.133, p_3=31.6) and in the TDI group 8 mg ACh/m³ air (p_1=75.6, p_2=-0.407, p_3=7.84). Despite the comparable test design the HDI-sensitzed animals did not show similar changes.

Figure 8: Acetylcholine bronchoprovocation assay. Stepped exposure of ACh: 0.1% - 0.2% - 0.4% - 0.8% (nebulized solution). TDI (II) = group 4



8.3. Elicitation of Respiratory Hypersensitivity by Conjugate Challenge

Guinea pigs were challenged with the respective hapten-conjugate of the haptens TDI or HDI in concentrations between 43 - 52 mg/m³ air. The conjugate aerosol had an MMAD ≈ 1.8

µm, GSD ≈ 1.9, and particle mass ≤ 3 µm of 80%. For more information see the Appendix.

The results obtained during or following challenge with the respective GPSA conjugate of the hapten are summarized in Table 3. All guinea-pigs tolerated the respective hapten conjugate challenge without specific clinical signs. The examination of delayed-onset responses (evaluated in four animals/group in groups 1' and 2' only) were inconclusive both after the hapten and after the conjugate challenges.

Table 3: Lung Function Measurements during Challenge with the respective Conjugate

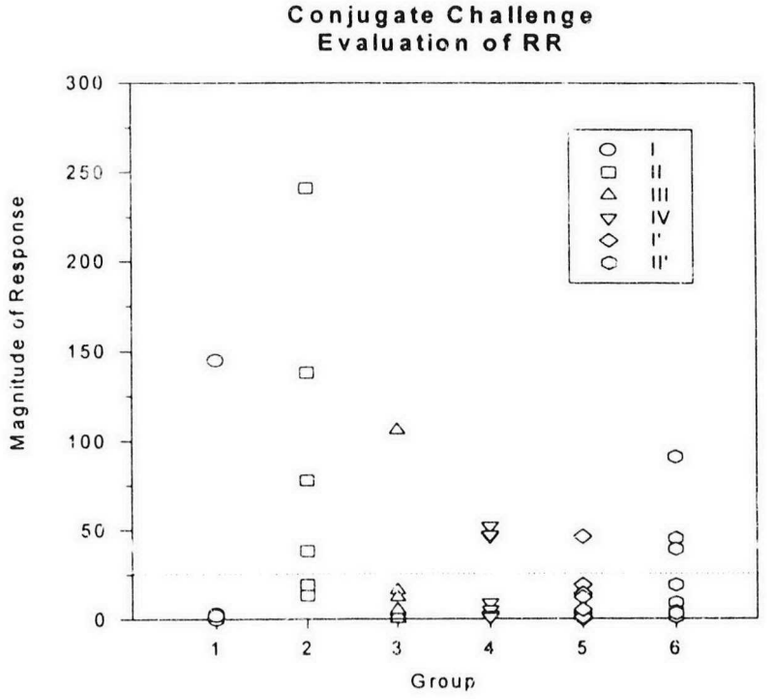
		A		B		C	
N	Regimen (target concentration)	RR	PEF x (IT+ET)/TV	RR	PEF x (IT+ET)/TV	RR	PEF x (IT+ET)/TV
1	vehicle	1/7	0/7	1/7	0/7	1/7	1/7
2	id - HDI	6/6	4/6	2/6	5/6	4/6	4/6
3	id + ih - HDI	5/8	6/8	2/8	5/8	1/8	4/8
4	id + ih - TDI	3/8	5/8	5/8	6/8	3/8	6/8
1'	vehicle	1/7	1/7	1/7	1/7	1/7	2/7
2'	id - TDI	4/8	3/8	3/8	3/8	3/8	3/8

Number of animals examined: 8 guinea pigs/group throughout the study, N = group no., A) Visual evaluation, B) Based on # of counts exceeding the mean of pooled pre-exposure data + 3STD, C) Based on # of counts exceeding the mean of individual pre-exposure data + 3STD

The type of data evaluation summarized in Table 3, column C 'RR' are depicted in Fig. 9.

Characteristic, stereotypic changes of lung function (increased respiratory rate), indicative of lung sensitization, were observed in both HDI and TDI-induced animals challenged with the respective hapten-GPSA conjugate. From Table 3 and Fig. 9 it is evident that both the incidence and the magnitude of response could not be increased by employing a combined intradermal - inhalation induction regimen. Comparison of the various modes of evaluation of data demonstrate that the most conclusive and less biased result was obtained when the number of counts based on individual rather than pooled pre-exposure data were evaluated.

Figure 9: Elicitation of respiratory allergy by TDI- and HDI- GPSA conjugate (# of counts < 25 considered to be not significantly different from controls, group assignment see box)



8.4. Necropsy and Histopathology

Gross pathological examinations showed roughly the same incidence of macroscopically apparent lung changes in all guinea pigs of this study. A list of the individual findings is included in the Appendix. Histopathological findings obtained in individual animals are itemized in Table 4. All findings are included in the respective Appendix.

From Table 4 it is evident that most characteristic findings related to the induction by inhalation is bronchiolitis. The influx of eosinophils into the main bronchi and LALN is taken as indirect evidence that TDI and HDI induced respiratory allergy.

Table 4: Summary of histopathological findings

N	Regimen (target concentration)	Lymphoid hyperplasia	EOS in Bronchi	EOS in LALN
1	vehicle	0/7	1/7	0/7
2	id - HDI	1/6	6/6**	5/6**
3	id + ih - HDI	2/8	3/8	5/8**
4	id + ih - TDI	7/8**	8/8**	4/8*
1'	vehicle	n.d.	n.d.	n.d.
2'	id - TDI	n.d.	n.d.	n.d.

Findings: moderate and severe combined, slight and very slight omitted, n.d.: not examined, * = $p < 0.05$, ** = $p < 0.01$ (Fisher's exact test, unilateral)

8.5. Serology

As summarized in Table 5, IgG_1-antibody determinations revealed high anti-hapten GPSA conjugate antibody titers both in animals sensitized to TDI and HDI. Details of this assay are reported in the Appendix.

Table 5: Summary of serological determinations

N	Regimen (target concentration)	Hapten-Conjugate dilution
1	vehicle	< 1:10
2	id - HDI	$1:10^4$
3	id + ih - HDI	$1:10^5$
4	id + ih - TDI	$1:10^5$
1'	vehicle	n.d.
2'	id - TDI	n.d.

n.d.: not determined

9. DISCUSSION AND ASSESSMENT

Following intradermal induction, skin irritation related reactions and during the inhalation induction, signs indicative of respiratory tract irritation occurred. During or following hapten-challenges, the incidence of immediate-onset respiratory reactions were roughly the same in all groups whereas during or following conjugat-challenges immediate-onset respiratory reactions occurred in a higher incidence in the HDI and TDI sensitized groups when compared to the control groups. The comparison of both routes of induction demonstrate that the incidence or magnitude of responding animals was not appreciably different when additional inhalation induction exposures were made. The acetylcholine bronchoprovocation challenge demonstrated that previous inhalation induction exposures to TDI, but not HDI, evoked a conspicous nonspecific bronchial hyperreactivity. The histopathological investigations revealed inflammatory responses in those groups receiving inhalation induction exposures (bronchiolitis). Independent of the route of induction, in both the HDI and TDI groups evidence of a specific airway eosinophilia and eosinophil infiltration into lung associated lymph nodes, a hallmark of allergic airway hyperresponsiveness, was observed. The serological investigations revealed a marked increase in anti-HDI/TDI IgG_1-antibody titres.

To summarize, when animals that were sensitized intradermally or by inhalation and were challenged by inhalation with the respective conjugate of the haptens conclusive immediate-onset responses were observed. Additional evidence of a lung sensitizing potential was provided by the histopathological examinations which revealed an increased eosinophilia of airways and lung associated lymph nodes as well as production of specific IgG_1-antibody. Therefore, this study provides clear evidence that HDI and TDI are a respiratory sensitizer in the guinea pig bioassay. It does not appear, in turn, that the combined intradermal and inhalation induction produces synergistic effects. These findings lend support the conclusion that successful induction and elicitation of allergic respiratory hypersensitivity can be achieved either by intradermal and by inhalation induction exposures. The first is easier to standardize and less prone to experimental artifacts.

10. KEY TO ABBREVIATIONS IN TABLES

MMAD	Mass Median Diameter
GSD	Geometric Standard Deviation
ECD	Effective cut-off diameter
STAND, S, Std, SD	Standard deviation (σ)
MW/MEANS, x	Means
B.W.	Body weight
F	F test value (F ratio)
DF	Degrees of freedom
PROB	Probability
SS	Total sum of squares
MS	Mean squares
TREATMENT	- Between the groups
ERROR	- Within the groups
TOTAL	- Total

Observation-No.: n-nn body weight gain from dates n to nn

11. REFERENCES

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ASTM - Standard Test Method for Estimating Sensory Irritancy of Airborne Chemicals. ASTM Designation: E 981-84. American Society for Testing and Materials, Philadelphia, USA.

BCTIC Computer Code Collection - Biomedical computing Technology Information Center, ANOVA a Fortran Program to Perform one-way Classification Analysis of Variance. Vanderbilt Medical Center, Nashville Tennessee, U.S.A.

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Characterization of Induction and Challenge Atmospheres

Nominal concentration - induction by inhalation

Date	HDI Concentration (mg/m³)	TDI Concentration (mg/m³)
13.11.1995	55.56	84.36
14.11.1995	36.11	84.35
15.11.1995	36.11	78.53
16.11.1995	33.33	69.81
16.11.1995	33.33	63.99
Mean ± STD	**38.9 ± 9.4**	**72.2 ± 9.1**

HDI: Day 0 Mean: 55.6, Day 1-4 Mean: 34.7

HDI induction by inhalation

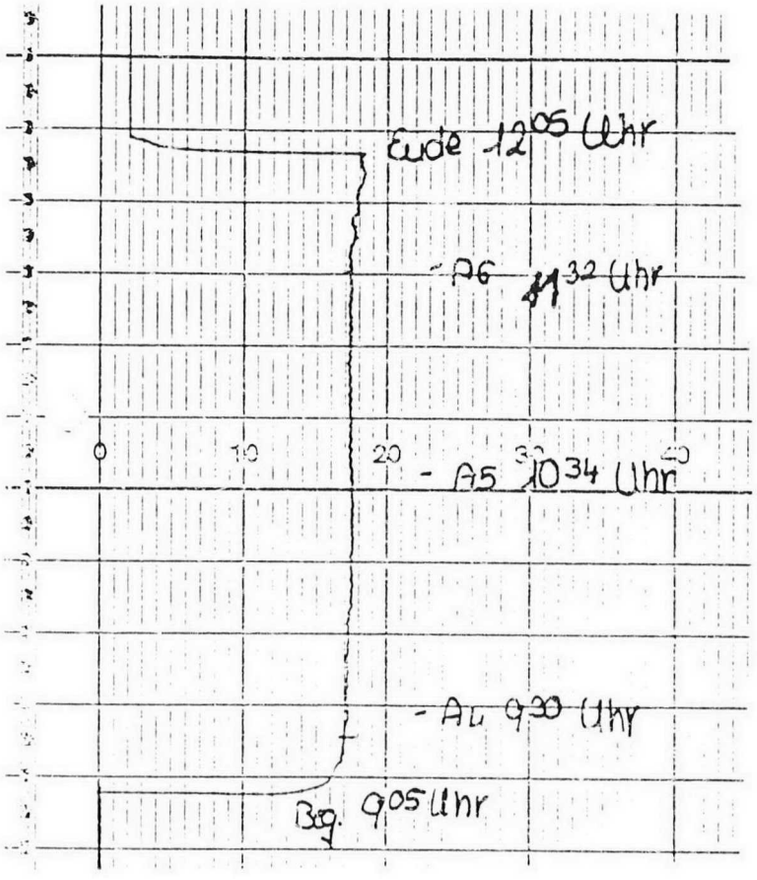
Date	Concentration (mg/m³)	Temperature (°C)	Relative Humidity (%)
13.11.1995	37.80 - 41.39 - 41.81	22.5	27
14.11.1995	21.04 - 24.89 - 25.39	22.6	15
15.11.1995	19.44 - 24.08 - 26.99	22.4	11
16.11.1995	21.20 - 24.54 - 27.22	22.5	15
16.11.1995	22.42 - 25.11 - 27.01	22.2	18
Mean ± STD	**27.4 ± 7.1**	**22.4 ± 0.2**	**17 ± 6**

HDI: Day 0 Mean ± STD: 40.3 ± 2.2, Day 1-4 Mean: 24.1 ± 2.6

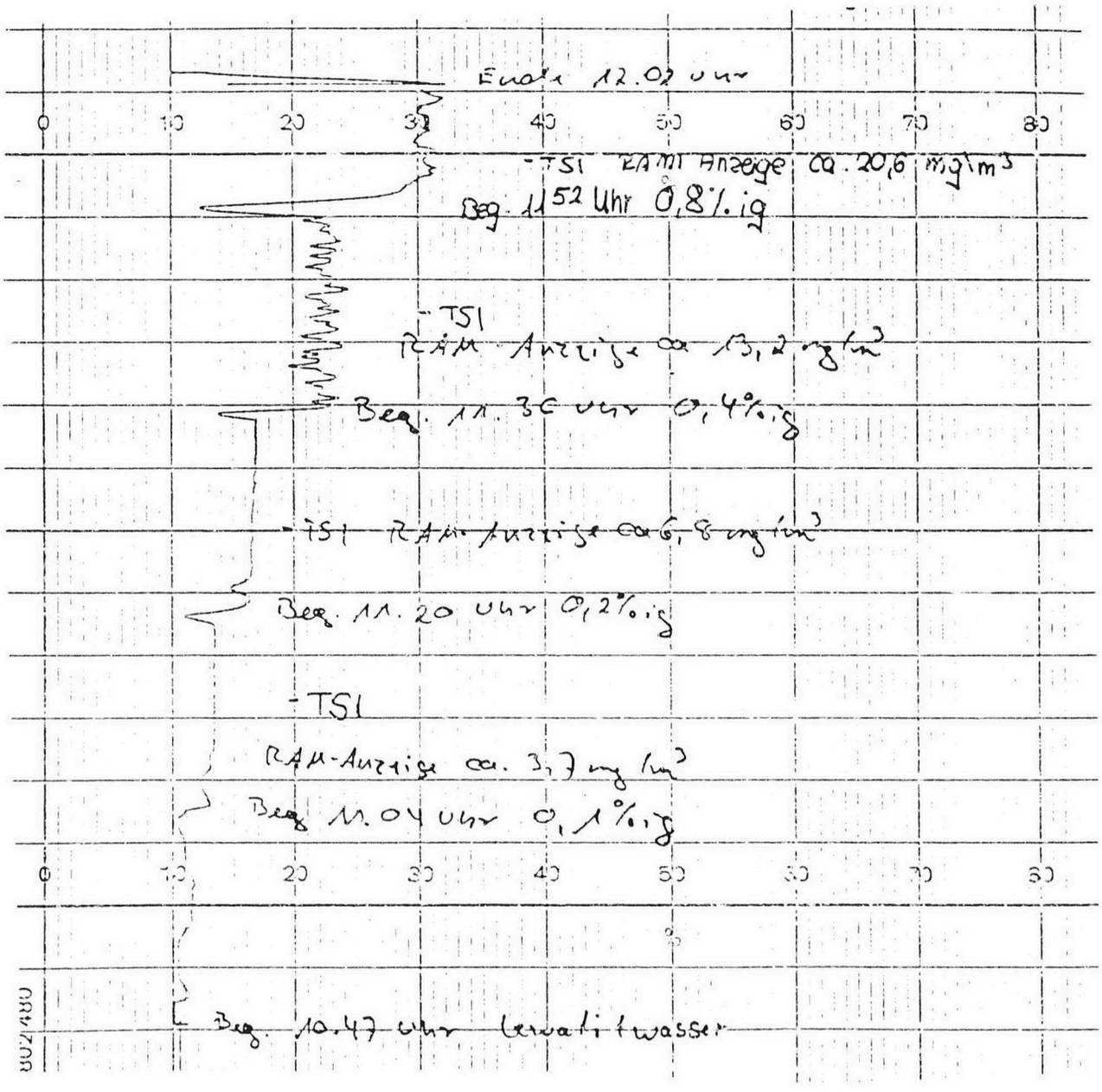
TDI induction by inhalation

Date	Concentration (mg/m³)	Temperature (°C)	Relative Humidity (%)
13.11.1995	50.75 - 54.29 - 54.24	21.8	28
14.11.1995	52.94 - 54.69 - 54.82	22.5	15
15.11.1995	47.68 - 50.41 - 50.47	22.3	15
16.11.1995	46.01 - 48.80 - 49.94	22.4	16
16.11.1995	51.02 - 52.10 - 52.10	22.4	14
Mean ± STD	**51.4 ± 2.6**	**22.3 ± 0.3**	**18 ± 6**

Monitoring of ACh-challenge atmospheres (example)



Monitoring of ACh-challenge atmospheres (example)



Particle-size determinations / ACh-challenge atmospheres

Challenge one day before hapten challenge

Group	ACh (%)	Equip-ment	MMAD (µm)	NMAD (µm)	GSD	% ≤ 3 µm	mg/m³ air
1	0.1	TSI	0.88	0.69	1.34	100	14.9
	0.2	TSI	0.86	0.66	1.34	100	15.5
	0.4	TSI	0.96	0.67	1.41	100	32.6
	0.8	TSI	1.02	0.66	1.46	100	75.2
2	0.1	TSI	0.87	0.67	1.35	100	13.9
	0.2	TSI	0.90	0.66	1.38	100	17.3
	0.4	TSI	0.98	0.67	1.43	100	34.6
	0.8	omitted					
3	0.1	TSI	0.80	0.62	1.34	100	4.3
	0.2	TSI	0.82	0.63	1.35	100	7.1
	0.4	TSI	0.93	0.65	1.41	100	19.8
	0.8	TSI	1.04	0.67	1.47	100	43.3
4	0.1	TSI	0.80	0.63	1.33	100	4.3
	0.2	TSI	0.84	0.63	1.36	100	8.3
	0.4	TSI	0.93	0.65	1.41	100	18.2
	0.8	TSI	1.01	0.66	1.45	100	40.8

Particle-size determinations / ACh-challenge atmospheres

Challenge one day after hapten challenge

Group	ACh (%)	Equip-ment	MMAD (µm)	NMAD (µm)	GSD	% ≤ 3 µm	mg/m³ air
1	0.1	TSI	0.84	0.64	1.35	100	9.1
	0.2	TSI	0.86	0.64	1.37	100	12.0
	0.4	TSI	0.92	0.64	1.42	100	21.2
	0.8	TSI	1.06	0.63	1.52	100	46.9
2	0.1	TSI	0.79	0.64	1.30	100	6.0
	0.2	TSI	0.84	0.64	1.35	100	10.8
	0.4	TSI	0.95	0.64	1.43	100	23.3
	0.8	TSI	1.00	0.66	1.45	100	39.1
3	0.1	TSI	0.79	0.61	1.34	100	4.8
	0.2	TSI	0.84	0.63	1.37	100	8.9
	0.4	TSI	0.93	0.64	1.42	100	20.3
	0.8	TSI	1.05	0.66	1.48	100	44.7
4	0.1	TSI	0.75	0.61	1.30	100	4.0
	0.2	TSI	0.32	0.63	1.35	100	8.6
	0.4	TSI	0.94	0.64	1.43	100	23.8
	0.8	TSI	1.01	0.66	1.46	100	39.0

Particle analyses (examples)

Particle-size determinations / ACh-challenge atmosphere - 0.1%

TSI AERODYNAMIC PARTICLE SIZER

Acetylcholin/T3060304 0.1 %ig

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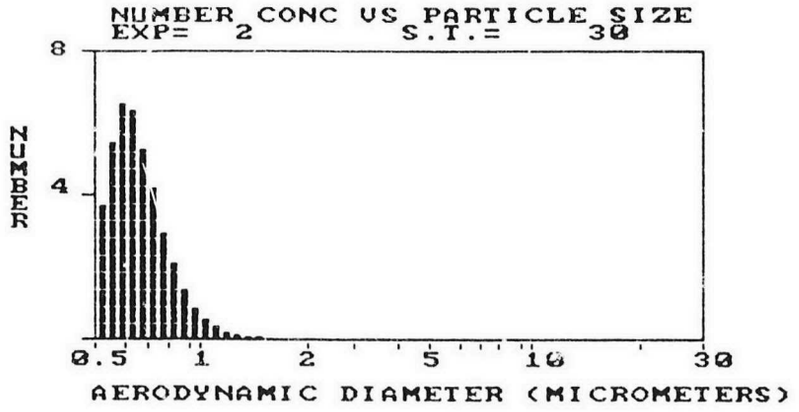
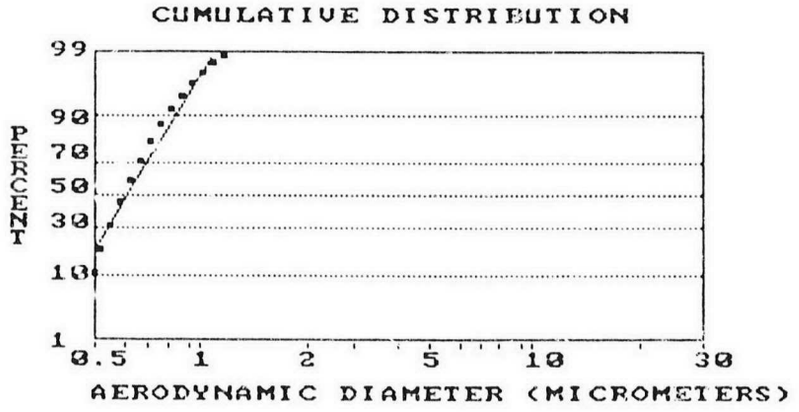
LAST CALIBRATION: 09-14-1995 SN 152





NUMBER MEDIAN DIAMETER (NMAD): 0.61 µm
MASS MEDIAN DIAMETER (MMAD): 0.75 µm
GSD : 1.30

MASS FRACTION < 3 µm : 100 PERCENT
PARTICLES PER cm³ : 4805.1
CONCENTRATION (COMPUTED) : 4.0 mg/m³

Particle-size determinations / ACh-challenge atmosphere - 0.2%

TSI AERODYNAMIC PARTICLE SIZER

Acetylcholin/T3060304 0.2 %ig

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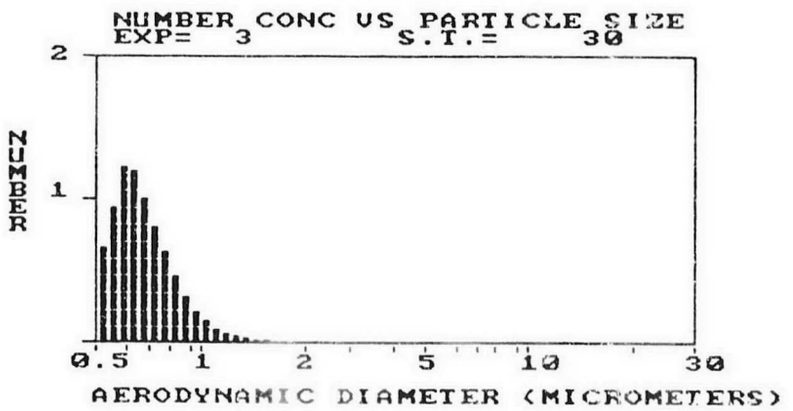

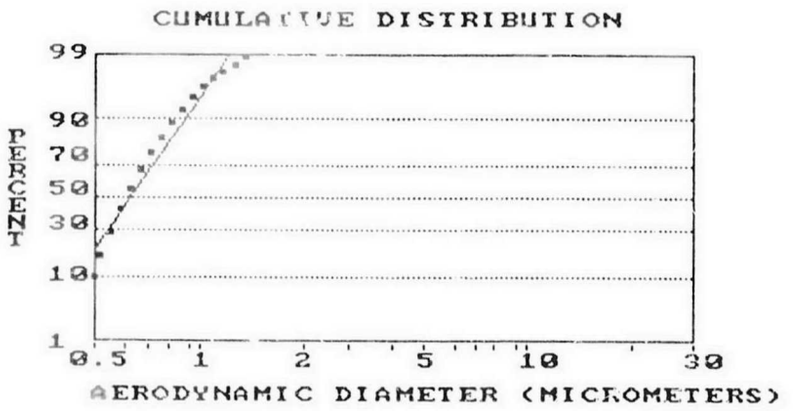

NUMBER MEDIAN DIAMETER (NMAD)	:	0.63 µm
MASS MEDIAN DIAMETER (MMAD)	:	0.82 µm
GSD	:	1.35
MASS FRACTION < 3 µm	:	100 PERCENT
PARTICLES PER cm³	:	8752.2
CONCENTRATION (COMPUTED)	:	8.6 mg/m³

BAYER AG T3060304

DEPARTMENT OF TOXICOLOGY 1,6-HEXAMETHYLENE DIISOCYANATE

Particle-size determinations / ACh-challenge atmosphere - 0.4%

TSI AERODYNAMIC PARTICLE SIZER

Acetylcholin/T3060304 0.4 %ig

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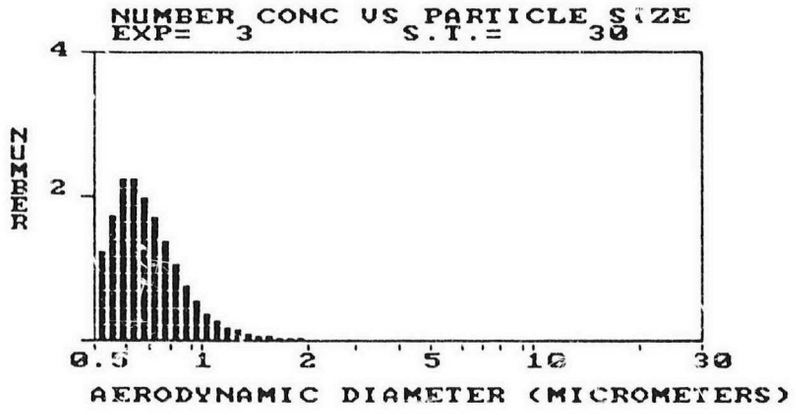
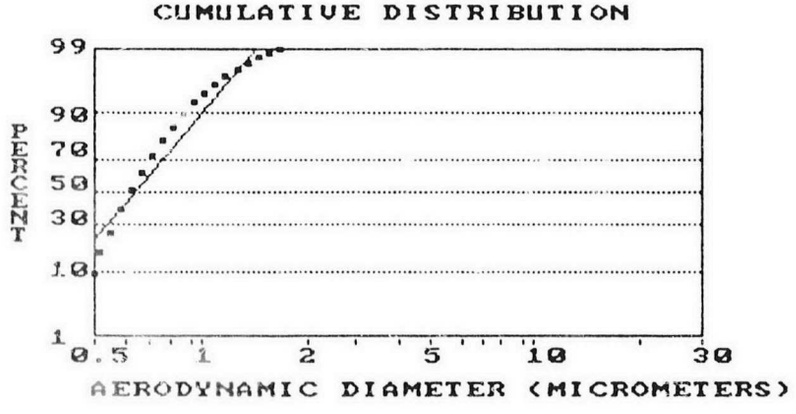
LAST CALIBRATION: 09-14-1995 SN 152





NUMBER MEDIAN DIAMETER (NMAD)	:	0.64 µm
MASS MEDIAN DIAMETER (MMAD)	:	0.94 µm
GSD	:	1.43
MASS FRACTION < 3 µm	:	100 PERCENT
PARTICLES PER cm³	:	17748.0
CONCENTRATION (COMPUTED)	:	23.8 mg/m³

BAYER AG T3060304
DEPARTMENT OF TOXICOLOGY 1,6-HEXAMETHYLENE DIISOCYANATE

Particle-size determinations / ACh-challenge atmosphere - 0.8%

TSI AERODYNAMIC PARTICLE SIZER

Acetylcholin/T3060304 0.8 %ig

SAMPLE # 1 DATE: 06.12.1995 SAMPLE TIME: 30 SEC DENSITY: 1
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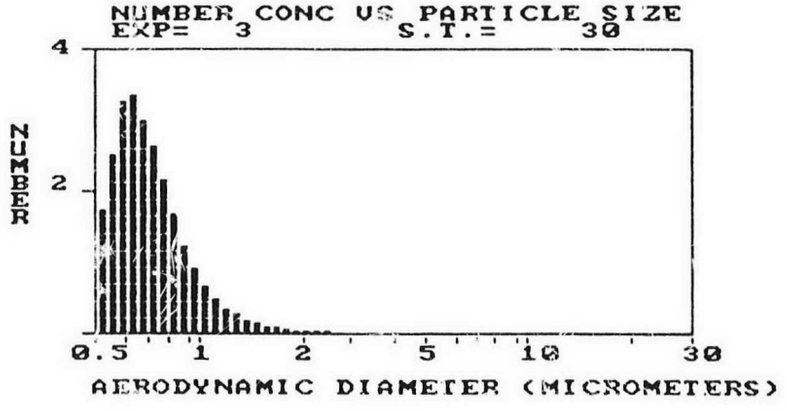
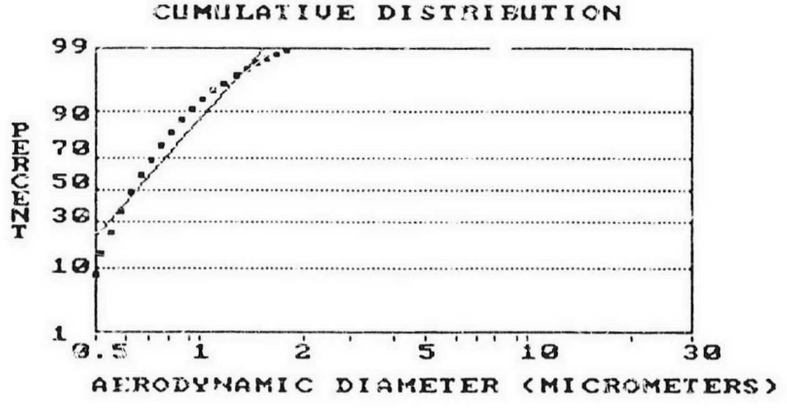
LAST CALIBRATION: 09-14-1995 SN 152





NUMBER MEDIAN DIAMETER (NMAD)	:	0.66 µm
MASS MEDIAN DIAMETER (MMAD)	:	1.01 µm
GSD	:	1.46
MASS FRACTION < 3 µm	:	100 PERCENT
PARTICLES PER cm³	:	27297.5
CONCENTRATION (COMPUTED)	:	39.0 mg/m³

Characterization of hapten and conjugate challenge atmospheres

Group	HDI Concentration (mg/m^3)	TDI Concentration (mg/m^3)	Conjugate Concentration (mg/m^3)
1	0.493	--	48
2	0.493	--	52
3	*	--	48
4	--	0.582**	52
Mean	0.5	0.6	50

*) Incorrect sampling probe, value were measured again approximately one month later without animals yielded lower concentrations despite duplicated exposure technology (0.093 and 0.112 mg/m^3 air). Since no plausible explanation for this discrepancy

**) Incorrect sampling probe, value were measured agian approximately one month later without animals

-- = not applicable

Particle-size distribution - HDI Conjugate

ANALYSIS OF PARTICLE DISTRIBUTIONS

Type of investigation: Acute Inhalation - Aerosol

Compound: HDI-Konjugat

Date of exposure: 30.10.95 Study-no.: T3060304
Concentration: 30.0 mg/m³ air

N	Impactor stage (µm - µm)	Cut-Off diameter (µm)	Mass/ stage (mg)	Rel. mass (%)	Cumul. mass (%)
1	0.06 - 0.12	0.06	.000	.00	.00
2	0.12 - 0.25	0.12	.017	.66	.00
3	0.25 - 0.49	0.25	.049	1.91	.66
4	0.49 - 0.90	0.49	.469	18.26	2.57
5	0.90 - 1.85	0.90	.986	38.40	20.83
6	1.85 - 3.69	1.85	.896	34.89	59.23
7	3.69 - 7.42	3.69	.150	5.84	94.12
8	7.42 - 14.8	7.42	.001	.04	99.96
9	14.8 - 30.	14.8	.000	.00	100.00

Mass Median Aerodynamic Diameter (MMAD): 1.51 µm
Geometric standard deviation: 1.79

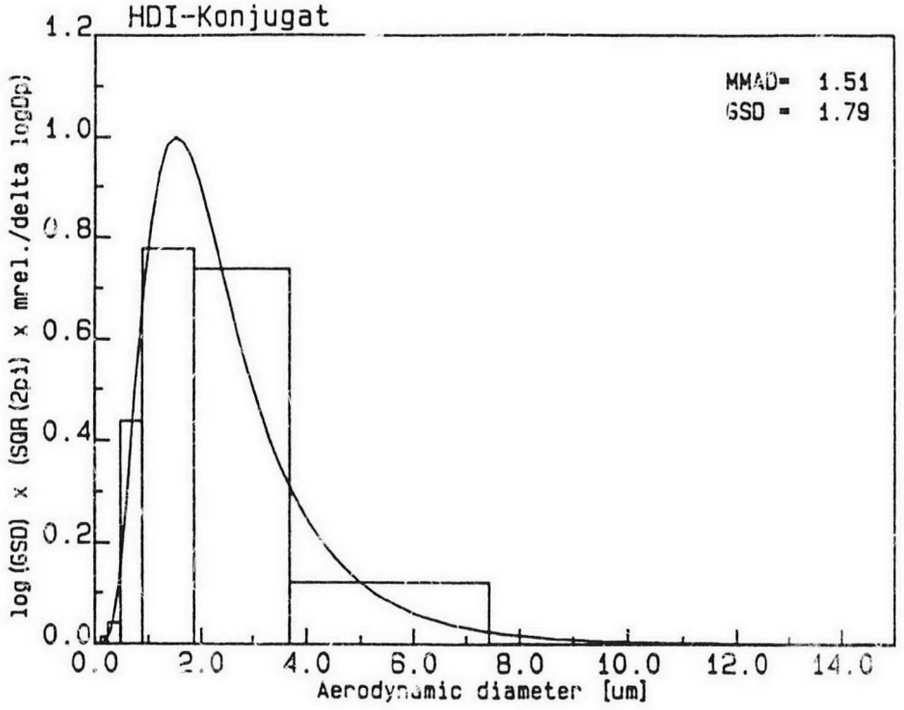
Number Median Aerodynamic Diameter (NMAD): .548 µm
Surface Median Aerodynamic Diameter (SMAD): 1.08 µm

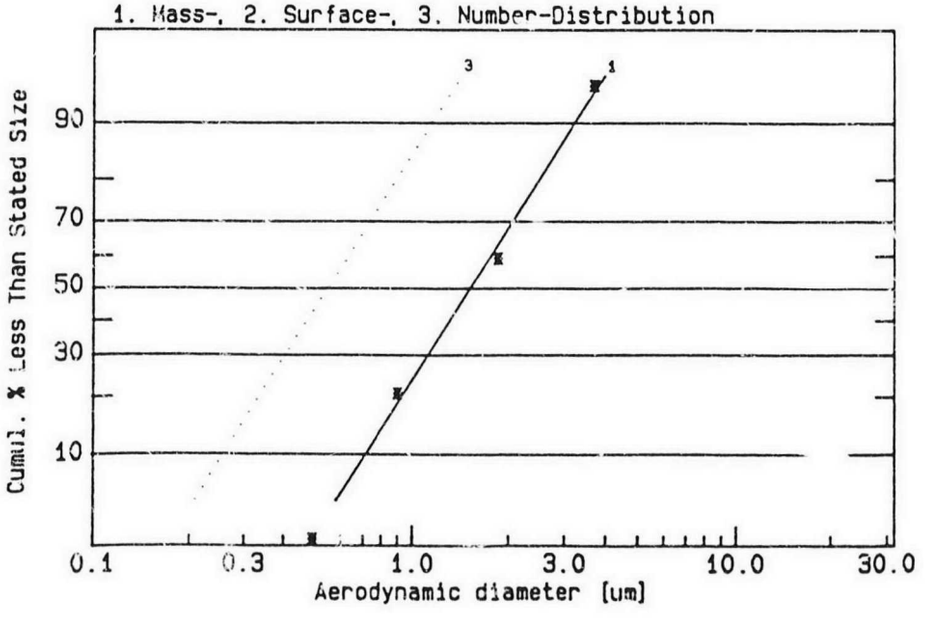
System: BERNER-IMPACTOR I
Air flow: 5.86 liter/min.
Sampling time: 600.00 seconds
Concentration (computed): 43.8 mg per m³ air

Respirability (% ≤ 3 µm):
1. Mass related: 88 % (measured)
2. Number related: 100 % (extrapolated)

EFFECTIVE CUT-OFF DIAMETER (ECD): The calculation of the cumulative distribution is based on the 'Effective Cut-Off Diameter'.

Acute Inhalation - Aerosol St.-no.: T3060304





Particle-size distribution - TDI Conjugate

ANALYSIS OF PARTICLE DISTRIBUTIONS

Type of investigation: Acute Inhalation - Aerosol

Compound: TDI-Konjugat

Date of exposure: 11.12.95 Study-no.: T3060304
Concentration: 30.0 mg/m³ air

N	Impactor stage (µm - µm)	Cut-Off diameter (µm)	Mass/ stage (mg)	Rel. mass (%)	Cumul. mass (%)
1	0.06 - 0.12	0.06	.000	.00	.00
2	0.12 - 0.25	0.12	.006	.28	.00
3	0.25 - 0.49	0.25	.040	1.86	.28
4	0.49 - 0.90	0.49	.244	11.33	2.14
5	0.90 - 1.85	0.90	.759	35.25	13.47
6	1.85 - 3.69	1.85	.851	39.53	48.72
7	3.69 - 7.42	3.69	.249	11.57	88.25
8	7.42 - 14.8	7.42	.004	.19	99.81
9	14.8 - 30.	14.8	.000	.00	100.00

Mass Median Aerodynamic Diameter (MMAD): 1.79 µm
Geometric standard deviation: 1.87

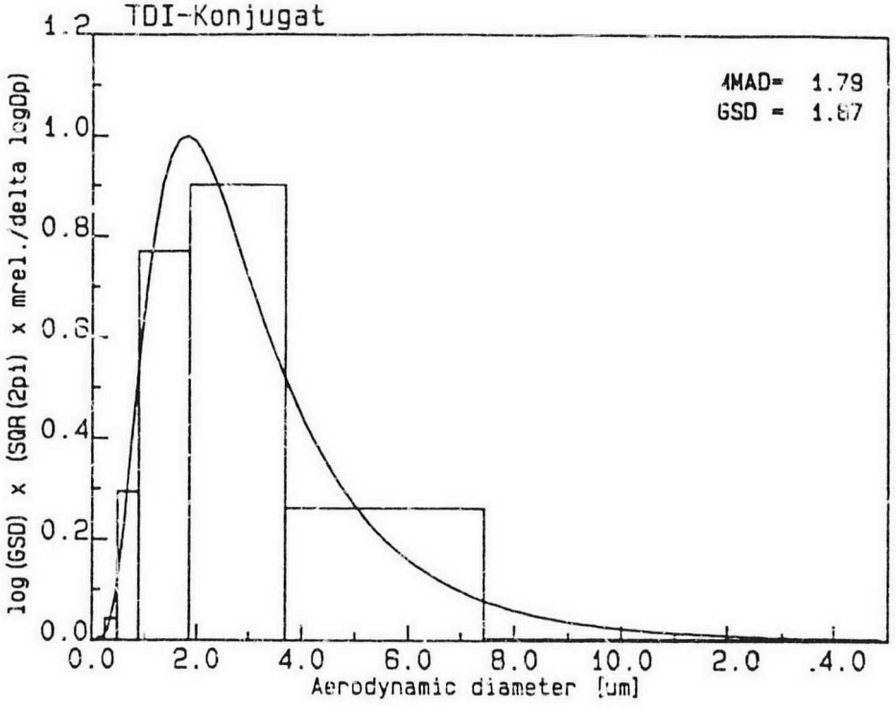
Number Median Aerodynamic Diameter (NMAD): .549 µm
Surface Median Aerodynamic Diameter (SMAD): 1.21 µm

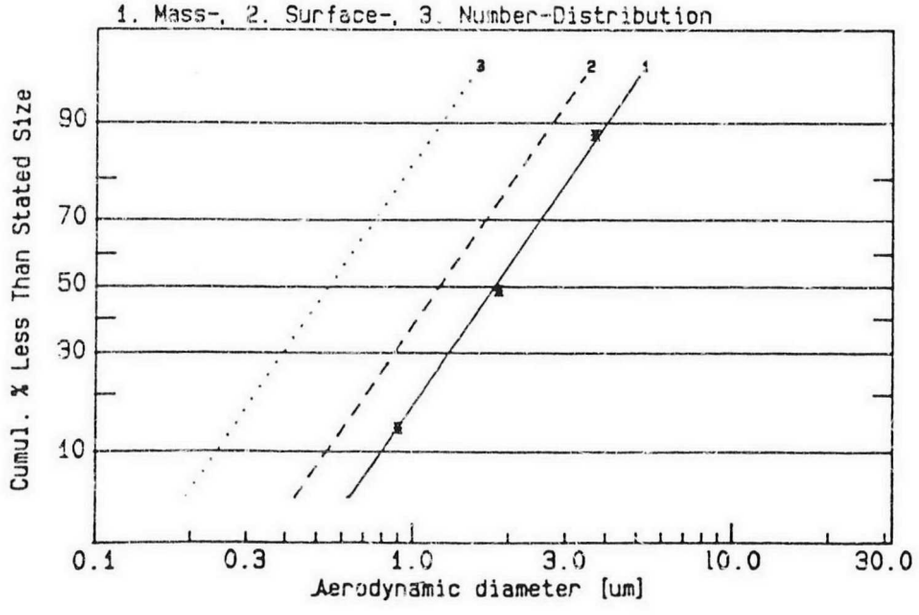
System: BERNER-IMPACTOR I
Air flow: 5.86 liter/min.
Sampling time: 600.00 seconds
Concentration (computed): 36.7 mg per m³ air

Respirability (% ≤ 3 µm):
1. Mass related: 80 % (measured)
2. Number related: 100 % (extrapolated)

EFFECTIVE CUT-OFF DIAMETER (ECD): The calculation of the cumulative distribution is based on the 'Effective Cut-Off Diameter'.

Acute Inhalation - Aerosol St.-no.: T3060304



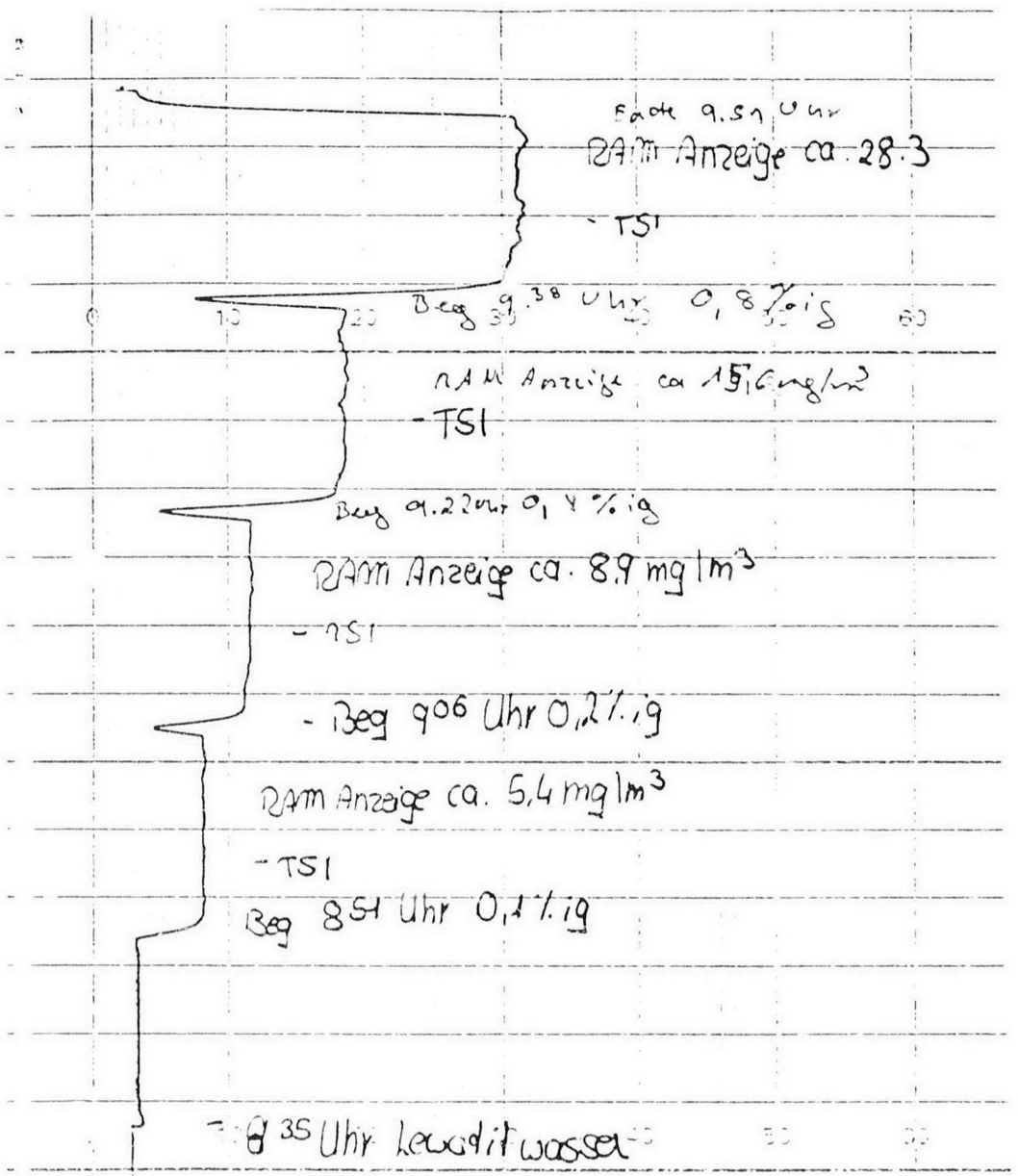


Characterization of Challenge Atmospheres

Characterization of hapten and conjugate challenge atmospheres

Group	TDI Concentration (mg/m^3)	TDI-Conjugate Concentration (mg/m^3)
1	0.06	43
2	0.042	43
Mean	0.05	43

Monitoring of ACh-challenge atmospheres (example)



Particle-size determinations / ACh-challenge atmospheres

Challenge one day before hapten challenge

Group	ACh (%)	Equip-ment	MMAD (µm)	NMAD (µm)	GSD	% ≤ 3 µm	mg/m³ air
1	0.1	TSI	0.76	0.62	1.29	100	3.4
	0.2	TSI	0.83	0.64	1.35	100	8.9
	0.4	TSI	0.93	0.65	1.41	100	19.7
	0.8	omitted					
2	0.1	TSI	0.79	0.63	1.31	100	3.7
	0.2	TSI	0.83	0.64	1.34	100	8.6
	0.4	TSI	0.92	0.65	1.40	100	18.2
	0.8	TSI	1.02	0.66	1.46	100	35.0

Challenge one day after hapten challenge

Group	ACh (%)	Equip-ment	MMAD (µm)	NMAD (µm)	GSD	% ≤ 3 µm	mg/m³ air
1	0.1	TSI	0.76	0.64	1.27	100	3.8
	0.2	TSI	0.83	0.65	1.32	100	9.9
	0.4	TSI	0.91	0.67	1.38	100	16.9
	0.8	TSI	0.97	0.68	1.41	100	34.6
2	0.1	TSI	0.74	0.63	1.26	100	4.0
	0.2	TSI	0.82	0.65	1.33	100	6.9
	0.4	TSI	0.88	0.66	1.36	100	17.0
	0.8	TSI	0.97	0.67	1.42	100	37.4

Particle analyses (examples)

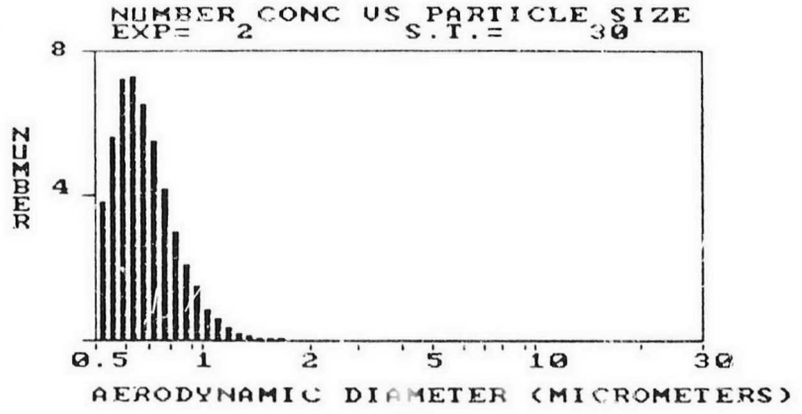
Particle-size determinations / ACh-challenge atmosphere - 0.1%

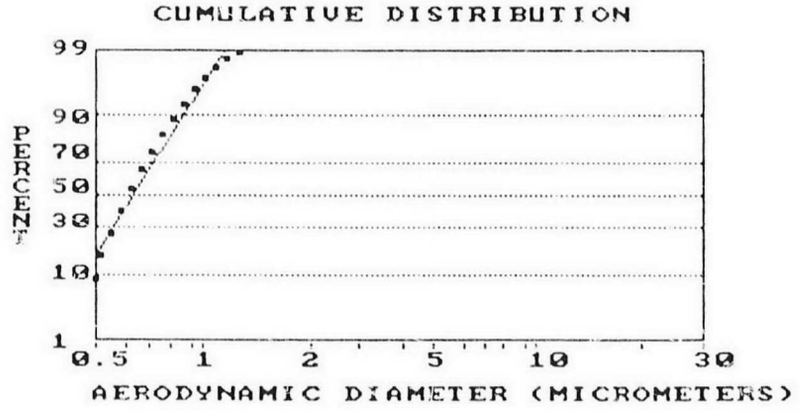
TSI AERODYNAMIC PARTICLE SIZER

Acetylcholin/T3060700 0.1 %ig

SAMPLE # 1 DATE: 28.02.1996 SAMPLE TIME: 30 SEC DENSITY: 1
DIL. RATIO: 100 :1 EFFIC. CORRECT.: D100 FILENAME: ace_2802.000
TIME: 09:19 OPERATOR: SPRU

LAST CALIBRATION: 09-14-1995 SN 152





NUMBER MEDIAN DIAMETER (NMAD)	:	0.63 µm
MASS MEDIAN DIAMETER (MMAD)	:	0.79 µm
GSD	:	1.31
MASS FRACTION < 3 µm	:	100 PERCENT
PARTICLES PER cm³	:	5375.0
CONCENTRATION (COMPUTED)	:	3.7 mg/m³

Particle-size determinations / ACh-challenge atmosphere - 0.2%

TSI AERODYNAMIC PARTICLE SIZER

Acetylcholin/T3060700 0.2 %ig

SAMPLE # 1 DATE: 28.02.1996 SAMPLE TIME: 30 SEC DENSITY: 1
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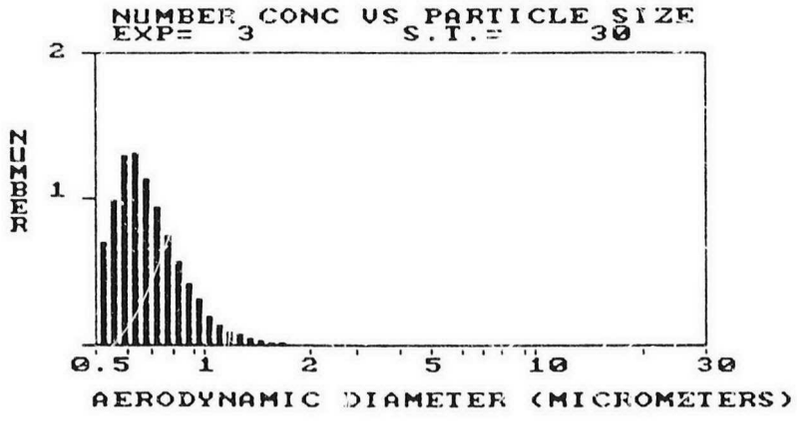

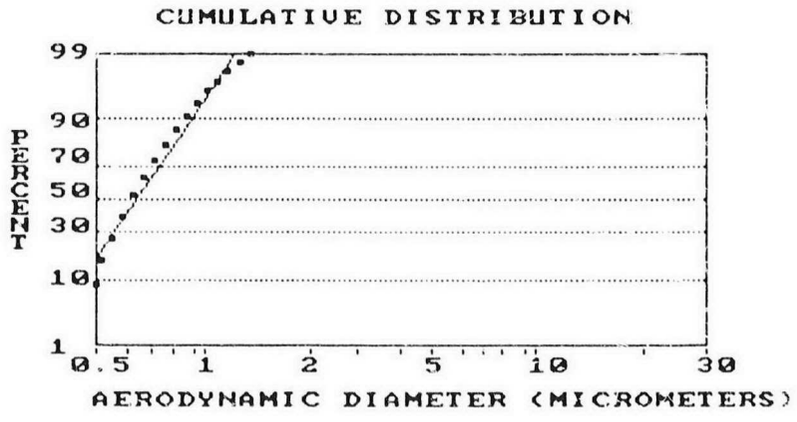

NUMBER MEDIAN DIAMETER (NMAD)	:	0.64 µm
MASS MEDIAN DIAMETER (MMAD)	:	0.83 µm
GSD	:	1.34
MASS FRACTION < 3 µm	:	100 PERCENT
PARTICLES PER cm³	:	9929.2
CONCENTRATION (COMPUTED)	:	8.6 mg/m³

BAYER AG
DEPARTMENT OF TOXICOLOGY

T3060700
TDI

Particle-size determinations / ACh-challenge atmosphere - 0.4%

TSI AERODYNAMIC PARTICLE SIZER

Acetylcholin/T3060700 0.4 %ig

SAMPLE # 1 DATE: 28.02.1996 SAMPLE TIME: 30 SEC DENSITY: 1
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TIME: 09:53 OPERATOR: SPRU

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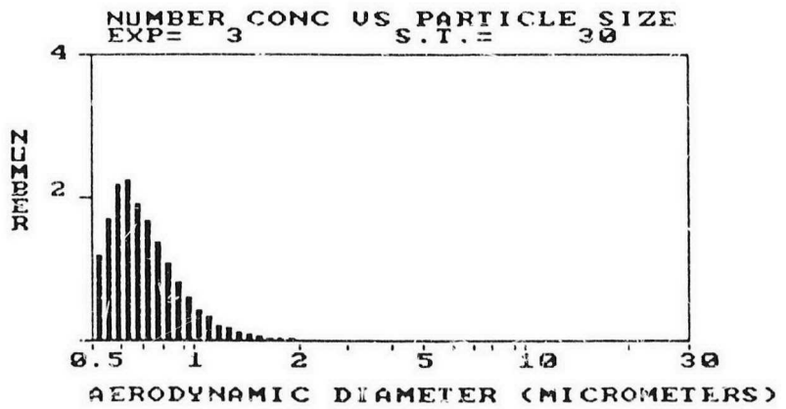
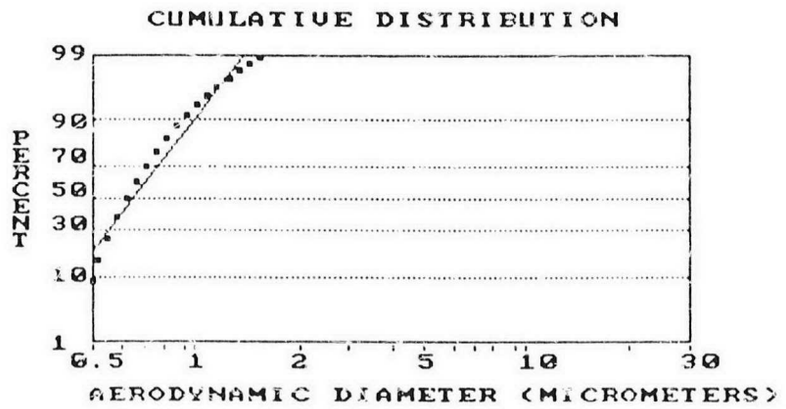




NUMBER MEDIAN DIAMETER (NMAD): 0.65 µm
MASS MEDIAN DIAMETER (MMAD): 0.92 µm
GSD : 1.40

MASS FRACTION < 3 µm : 100 PERCENT
PARTICLES PER cm³ : 17844.1
CONCENTRATION (COMPUTED) : 18.2 mg/m³

BAYER AG
DEPARTMENT OF TOXICOLOGY

T3060700
TDI

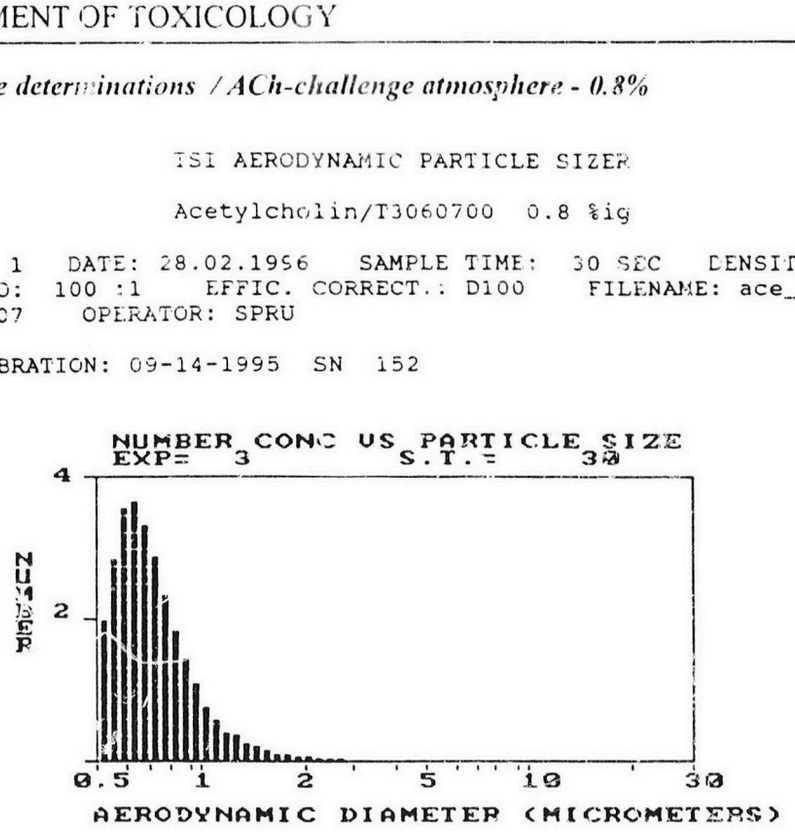
Particle-size determinations / ACh-challenge atmosphere - 0.8%

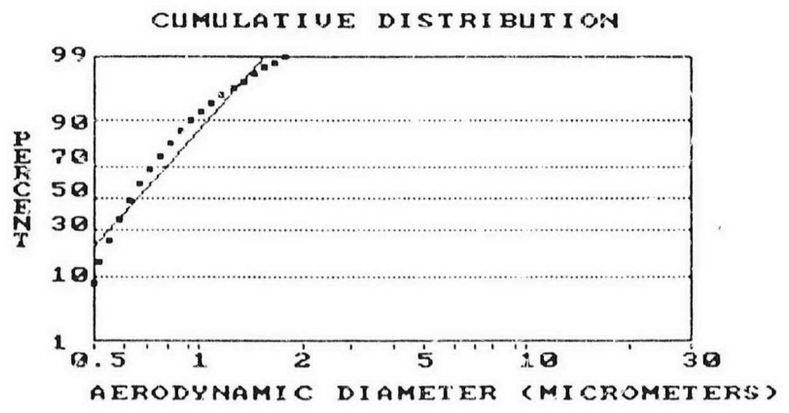
TSI AERODYNAMIC PARTICLE SIZER

Acetylcholin/T3060700 0.8 %ig

SAMPLE # 1 DATE: 28.02.1996 SAMPLE TIME: 30 SEC DENSITY: 1
DIL. RATIO: 100 :1 EFFIC. CORRECT.: D100 FILENAME: ace_2802.003
TIME: 10:07 OPERATOR: SPRU

LAST CALIBRATION: 09-14-1995 SN 152





NUMBER MEDIAN DIAMETER (NMAD): 0.66 µm
MASS MEDIAN DIAMETER (MMAD): 1.02 µm
GSD : 1.46

MASS FRACTION < 3 µm : 100 PERCENT
PARTICLES PER cm³ : 30541.2
CONCENTRATION (COMPUTED) : 35.0 mg/m³

Particle-size distribution - TDI Conjugate

ANALYSIS OF PARTICLE DISTRIBUTIONS

Type of investigation: Acute Inhalation - Aerosol

Compound: TDI-Konjugat

Date of exposure: 05.03.96 Study-no.: T3060700
Concentration: 30.0 mg/m³ air

N	Impactor stage (µm - µm)	Cut-Off diameter (µm)	Mass/ stage (mg)	Rel. mass (%)	Cumul. mass (%)
1	0.06 - 0.12	0.06	.000	.00	.00
2	0.12 - 0.25	0.12	.002	.10	.00
3	0.25 - 0.49	0.25	.057	2.96	.10
4	0.49 - 0.90	0.49	.222	11.51	3.06
5	0.90 - 1.85	0.90	.580	30.08	14.57
6	1.85 - 3.69	1.85	.755	39.16	44.66
7	3.69 - 7.42	3.69	.309	16.03	83.82
8	7.42 - 14.8	7.42	.003	.16	99.84
9	14.8 - 30.	14.8	.000	.00	100.00

Mass Median Aerodynamic Diameter (MMAD): 1.90 µm
Geometric standard deviation: 2.03

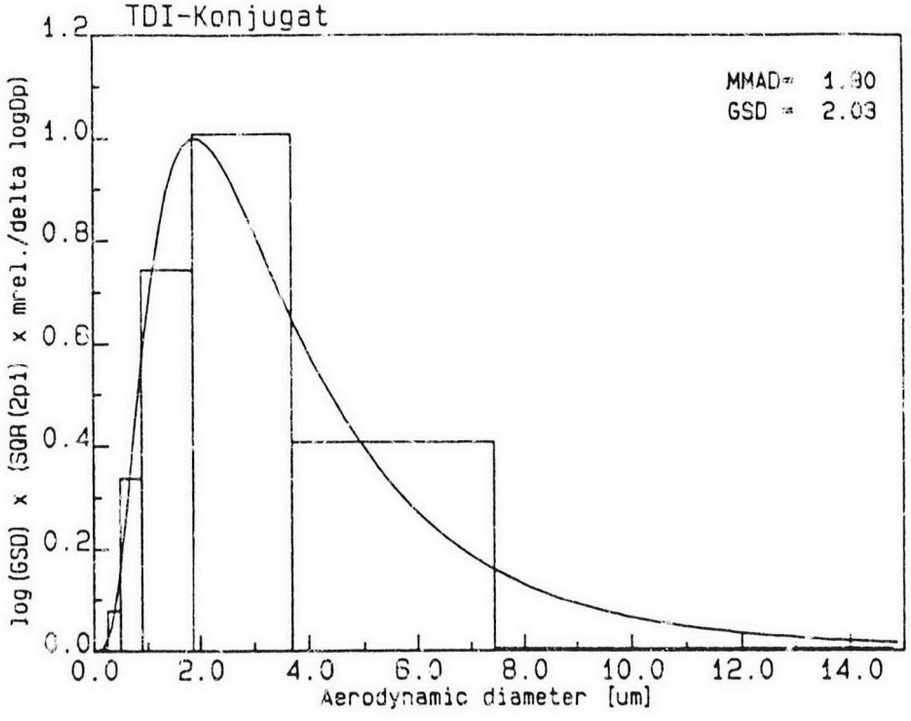
Number Median Aerodynamic Diameter (NMAD): .421 µm
Surface Median Aerodynamic Diameter (SMAD): 1.15 µm

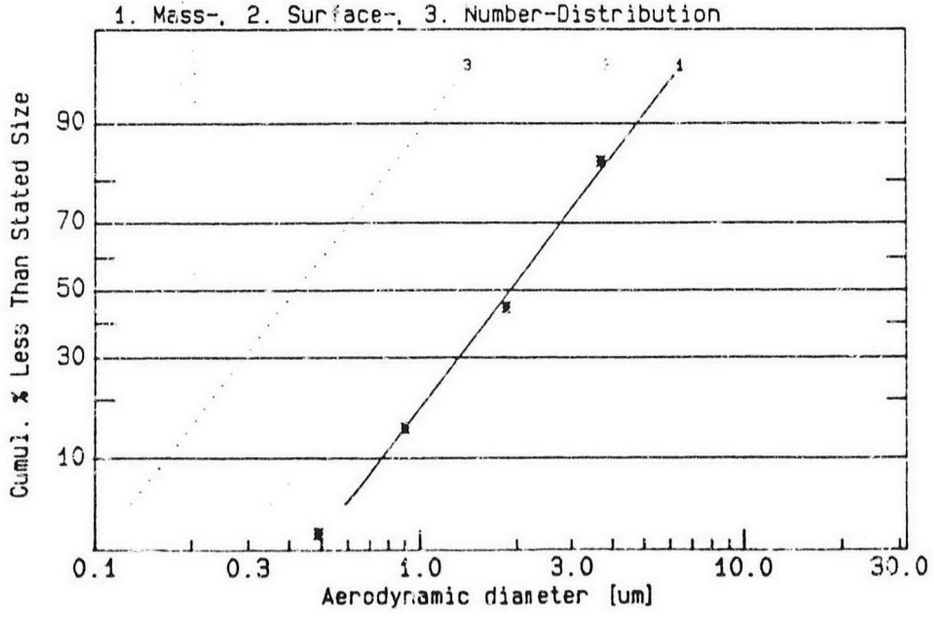
System: BERNER-IMPACTOR I
Air flow: 5.65 liter/min.
Sampling time: 600.00 seconds
Concentration (computed): 34.1 mg per m³ air

Respirability (% ≤ 3 µm):
1. Mass related: 74 % (measured)
2. Number related: 100 % (extrapolated)

EFFECTIVE CUT-OFF DIAMETER (ECD): The calculation of the cumulative distribution is based on the ´Effective Cut-Off Diameter´.

Acute Inhalation - Aerosol St.-no.: T3060700





Appendix - Analytical Characterization of Test Atmosphere

BAYER AG
DEPARTMENT OF TOXICOLOGY
FRIEDRICH-EBERT-STR. 217-333
D-42096 WUPPERTAL

DESMODUR H (HDI)

ANALYTICAL METHOD VALIDATION FOR

CONCENTRATION DETERMINATIONS IN TEST ATMOSPHERES

Analytical Report

Dr. W. Rüngeler

Study-No.: T3060304

1. TABLE OF CONTENTS

2. SUMMARY

An analytical method is described that can be used to determine the concentration of the test material based on Desmodur H (HDI) in test atmospheres.

The test material as a vapor is adsorbed on glass powder loaded with N-4-Nitrobenzyl-N-n-propylamine solution (nitro reagent). The isocyanate component reacts to form the corresponding urea derivative. After desorption with acetonitrile, the reaction product is quantified by high-performance liquid chromatography (HPLC; UV detection).
Standard solutions of the test material treated similary to test samples with the nitro reagent were used as basis for evaluation.

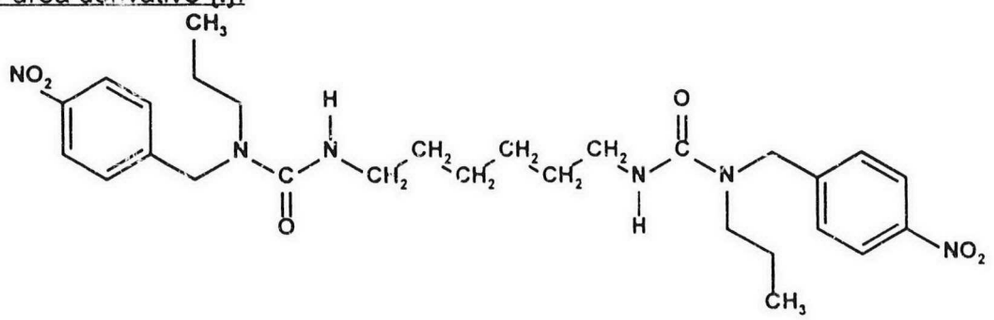
With a 20 litres atmosphere sample and an end solution volume of 25 ml, the limit of quantification for this test substance has been found to be **0.21 mg test material/m^3**.

3. INTRODUCTION

An analytical method for the quantification of 1,6-Hexamethylenediisocyanate (HDI) from test atmospheres was developed. The HDI was the basis of the analytical concentration determinations. This work was conducted in preparation for investigations on the inhalation toxicity of this test material. The method and its validation is described in this report.

In this method, developed by N. Kuck and modified by ourselves, the test material as a vapor is allowed to react with N-4-nitrobenzyl-N-n-propylamine (nitro reagent) to form the corresponding urea compound (I), which is then determined by high-performance liquid chromatography (HPLC) with UV detection. The test material vapor is adsorbed from the test atmosphere in two series-connected tubes packed with glass powder loaded with the nitro reagent solution. The HDI urea derivative (I) was then desorbed with acetonitrile and the solution was injected, after appropriate dilution, onto the HPLC.
Standard solutions of the test material treated similary to test samples with the nitro-reagent were used as basis for evaluation.

HDI-urea derivative (I):



Investigations necessary for drafting the Standard Operating Procedure and performing the analyses were conducted in September/October 1995 at the Institute of Industrial Toxicology, Department of Toxicology of Bayer AG, D-42096 Wuppertal-Elberfeld, Friedrich-Ebert-Strasse 217-333.
The study documentation (raw data and final analytical report) has been archived in locations specified by Bayer AG, in accordance with GLP requirements.

Study-No.: T3060304

4. MATERIALS AND METHOD

4.2. HIGH PERFORMANCE LIQUID CHROMATOGRAPHY

4.2.1. Apparatus

High performance liquid chromatograph HP1090 equipped with **
- Autosampler
- DAD (diode array detector)
- Integration: HP 3365 DOS-WorkStation/ChemSever **
supplied from Hewlett-Packard

4.2.2. Method

Column:	LiChrospher RP 18	5 µm; L: 125 mm; ID: 4mm; Merc. **
Oven temperature:		off
Mobile phase:	A:	50% buffer solution
	B:	50% acetonitrile
	gradient program:	time 3 min: 50%B --> time 6 min: 85%B
	buffer composition:	2 ml H_3PO_4 + 4 ml TEA ad 1000 ml Milli-Q-water
Flow rate:		1.0 ml/min
Injection volume:		20.0 µl
Detector:	wavelength:	275 nm
	band width (BW):	4 nm
	reference:	450 nm / 80 nm BW **

4.3. OTHER APPARATUS

Gas measuring device (Elster) **
Mini A-Pump (P) (Leybold-Heraeus) **
Rotameter (R) **
Manometer (D) **
Needle valve (V)
calibrated thermometer for temperature measurement
calibrated barometer
Standard laboratory equipment and glassware

small adsorption tubes with ground-glass joints (L = 120 mm, ID = 12 mm)
Packing: each tube 4 g glass powder
small adsorption tubes with ground-glass joints (L = 65 mm, ID = 12 mm)
Packing: each tube 2 g glass powder

Gas tight syringes (25 µl; 100 µl; 250 µl; 10 ml ; Hamilton) **

** or equivalent

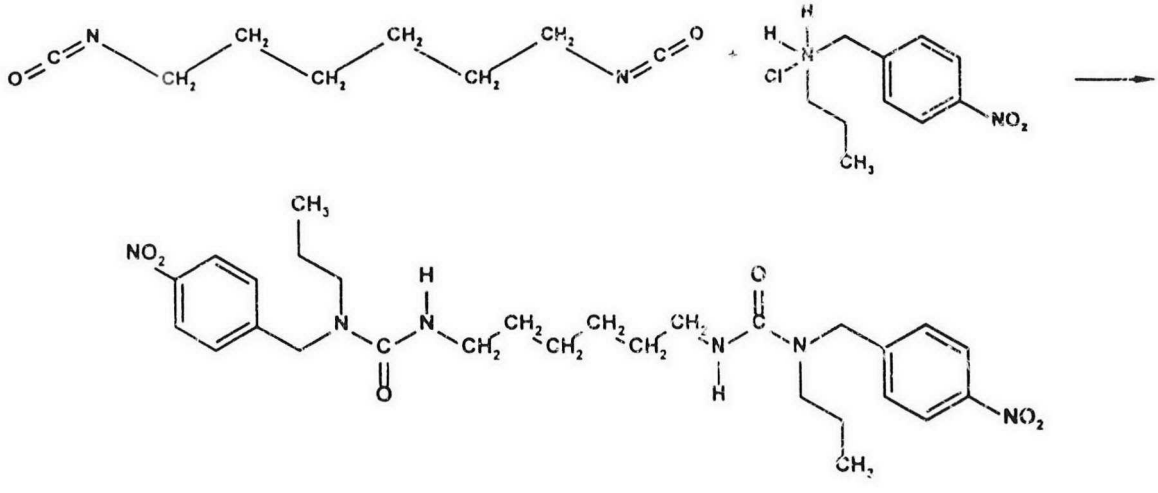
(The apparatus is regulary maintained and calibrated.)

4.4. SOLVENTS AND CHEMICALS

Acetonitrile p.a.; Merck
Deionized water (Milli-Q-water), Millipore unit
Dichloromethane p.A., Merck
N-4-Nitrobenzyl-N-n-propylammonium chloride p.A., Fa. Riedel de Haen, No. 33487
Glass powder 40/60 mesh; G. Karl, Part-No. GK 26-48004
sodium sulfate p.A., Merck
o-Phosphoric acid (85%ig); H_3PO_4 ; Merck
Triethylamine (TEA) ; Merck

4.4.1. Nitro reagent solution (absorption solution)

1.6 g N-4-nitrobenzyl-N-n-propylammonium chloride (corresponding to 1.34 g free base) is dissolved in 100 ml of deionized water and 50 ml of 1 N sodium hydroxide solution is added. A white precipitate (free base) is formed. The aqueous suspension is transferred into a 500 ml separating funnel and extracted with 250 ml dichloromethane. The organic phase is separated off, dried over sodium sulfate, transferred into a 500 ml volumetric flask, and made up to the mark with dichloromethane. This solution contains 2.7 mg nitro reagent (free base)/ml dichloromethane. The solution can be used as an absorption solution in impinger-flasks as well as for sample collection with glass powder-packed tubes, the nitro reagent serving to load the adsorbent carrier material.



empirical formula of the urea derivative: $C_{28}H_0N_6O_6$
The structure of the reaction product formed from HDI and nitro reagent is shown in the above equation. This urea derivative is analyzed in the HPLC (UV-detection) and is quantified, after recalculation, as free HDI.

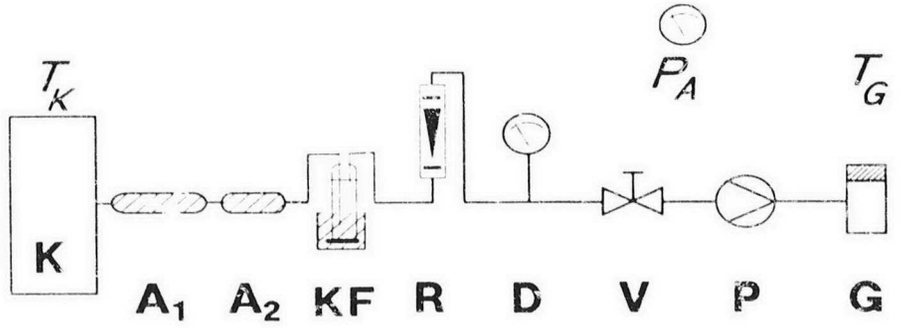
4.4.2. Calibration standards

30-50 mg of the test material is pipetted into a 50 ml volumetric flask and accurately weighed. The flask is then brought up to volume with nitro reagent solution (concentration: 2.7 mg/ml). Comparison standards in the desired concentrations are prepared from this solution by dilution with dichloromethane or acetonitrile.

5. SAMPLE COLLECTION AND PREPARATION

The surface of the glass powder in each adsorption tube is first loaded with 1 ml of the nitro reagent solution. The solvent is collected and discarded. Two series-connected adsorption tubes pretreated in the described way (A_1: 4g; A_2: 2g) are connected to the sampling apparatus (air throughput 0.5 to 1.0 l/min) (Fig. 2). The total volume of sampled air (V_X) the temperature of the gas flowmeter (T_G) the chamber temperature (T_K) and the barometric pressure (P_A) are recorded. After the end of the sample collection adsorption tubes (A_1, A_2) are mounted against the flow direction on a 50 ml volumetric flask. To desorb the urea derivative a funnel is fitted and 45 ml of acetonitrile is passed slowly through the tubes. The contents of the volumetric flask are then made up to the mark with acetonitrile. Samples of low concentrations (approx. <5 mg/m^3) are eluted with 25 ml of acetonitrile. Solutions are then injected onto the HPLC after appropriate dilution.

Figure 2: Sample collection apparatus



K	Inhalation chamber	V	Needle valve
A_1	Adsorption tube; packing: 4 g glass powder	P	Pump
A_2	Adsorption tube; packing: 2 g glass powder	T_G	Temperature of Gas flow meter
KF	condenser (optional)	T_K	Temperature of chamber
R	Rotameter	P_A	Barometric pressure
D	Manometer	G	Gas flow meter

6. CALIBRATION OF THE ANALYTICAL METHOD

To set up the calibration series, test material solutions in nitro reagent solution were prepared with appropriate concentrations (see 4.4.2.). Method-specific adjustments were made on the HPLC and 20.0 µl of each calibration concentration was injected for preparation of the calibration curve.

Measurement wavelength: 275 nm (see the UV spectrum, Fig. 3).

Fig. 4 shows a typical chromatogram of these external calibration solutions. A statistically evaluated calibration curve is shown in Fig. 5. This curve was plotted by the integrator and was based upon the injected concentrations. The calibration curve was plotted anew for each analysis sequence, and deviations from this calibration range were therefore possible. All sample concentrations are always within the calibration range documented for each sample sequence. The quantitative evaluation was performed by determination and comparing the peak area of **HDI urea derivative** of the analytical solution with the peak areas of the external standard solutions.

Retention time: **2,4-HDI urea derivative** about 6.5 min **conc. range:** 0.492 to 98.4 µg/ml

Figure 3: UV spectra of the HDI urea derivative

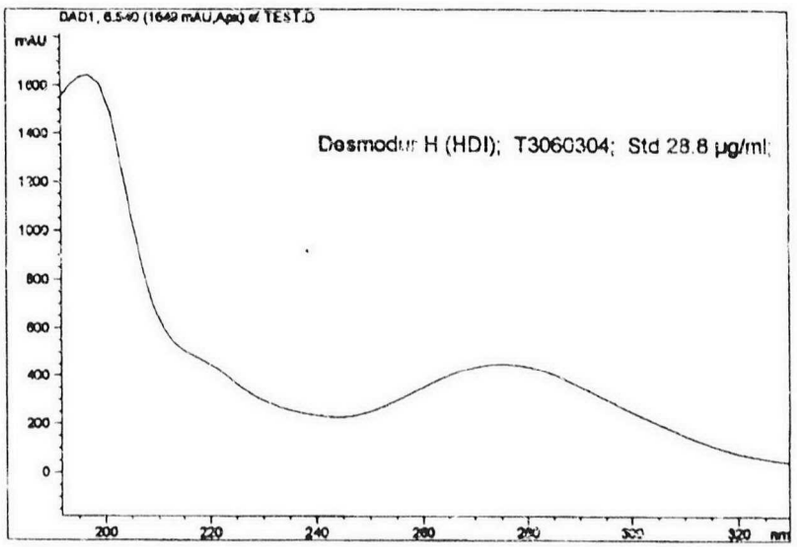


Figure 4: typical LC-chromatogram of the test substance urea derivative (calibration standard)
test material concentration: 28.8 µg/ml

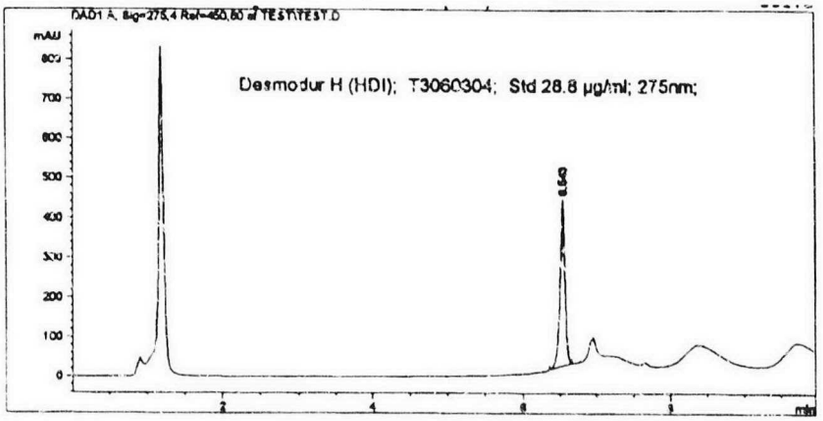
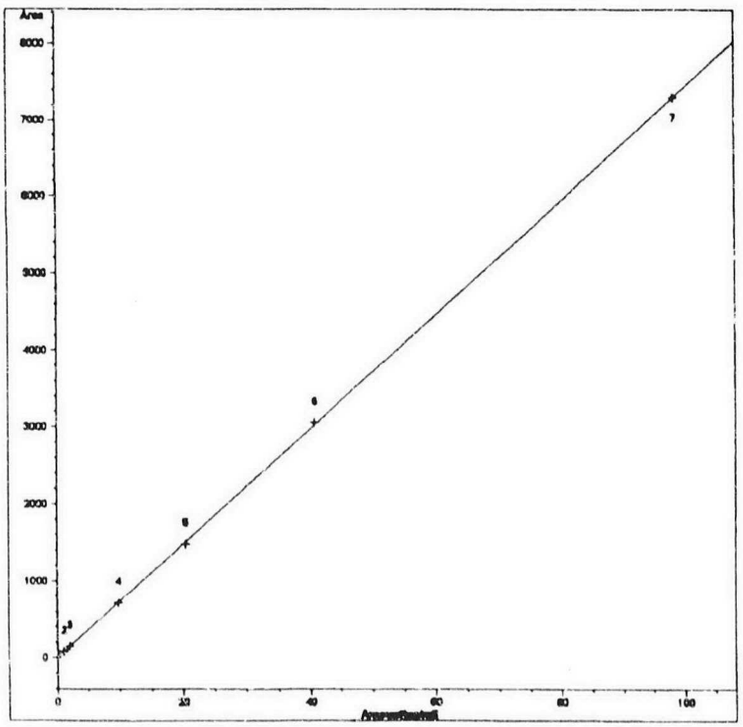


Figure 5: Calibration curve of the analytical method date: Aug. 23, 1995



The calibration is linear in the ranges shown. The linear regression value is $r^2 = 0.99998$.

7. CALCULATION OF THE ANALYTICAL RESULTS

Each sample within a sequence was injected twice. Since the sample and standard are treated identically, the concentration results do not need to be recalculated. The integrator evaluates each sample based on the plotted external standard calibration curve (see section 6.). The results are expressed in units of µg test material/ml solution.
The test material concentration in the test atmosphere is determined from the relationship:

$$\text{mg test material} / m^3 \text{ air} = \frac{X * F}{V_X} * \frac{(273 + T_G)}{(273 + T_K)}$$

F		dilution factor (= 25/50 for undiluted analytical solution)
X	[µg/ml]	test material concentration in the analytical solution
V_X	[l]	chamber atmosphere collected volume
T_G	[°C]	temperature of the gas flowmeter
T_K	[°C]	temperature of the inhalation chamber

8. STABILITY

The stability of Desmodur H in acetonitrile/dichloromethane was checked at room temperature over a period of 9 days.All solutions tested were found to be stable. No decrease in concentration was observed. The chromatographic sample preparation (elution of test material from glass powder, dilution, and injection) all are inducted during the tested time frames.

9. PRECISION

The precision of this analytical method was assessed by 10 separate injections for each of two relevant concentrations of the calibration standards. The area values obtained are presented in table 1. The precision of this method was found to satisfy the analytical requirements.
Table 1:

0.492 [µg/ml]	98.400 [µg/ml]
0.585	99.194
0.593	98.673
0.569	97.486
0.605	98.911
0.612	97.468
0.558	98.734
0.572	97.057
0.593	98.434
0.583	98.246
0.577	97.662
MEAN = 0.585	**MEAN = 98.187**
C_V = 2.8%	**C_V = 0.7%**

10. RECOVERY

The recovery was not specifically tested. The analytical results were compared with the parametric measurements in the actual study and were found to be consistent.

11. DETECTION LIMIT

The lowest detection limit of this analytical method is 0.492 µg test material/ml in acetonitrile. With a sample collection volume of 60 litres and an end dilution volume of 25 ml, a concentration of **0.21 mg HDI/m^3** can be accurately determined.

12. LITERATURE

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End of Report

Appendix - Analytical Characterization of Test Atmosphere

BAYER AG
DEPARTMENT OF TOXICOLOGY
FRIEDRICH-EBERT-STR. 217-333
D-42096 WUPPERTAL

DESMODUR T80 (TDI)

ANALYTICAL METHOD VALIDATION FOR

CONCENTRATION DETERMINATIONS IN TEST ATMOSPHERES

Analytical Report

Dr. W. Rüngeler

Study-No.: T3060700

0007

1. TABLE OF CONTENTS

2. SUMMARY

An analytical method is described that can be used to determine the concentration of the test material based on 2,4-TDI in test atmospheres.

The test material as a vapor is adsorbed on glass powder loaded with N-4-Nitrobenzyl-N-n-propylamine solution (nitro reagent). The isocyanate component reacts to form the corresponding urea derivative. After desorption with acetonitrile, the reaction product is quantified by high-performance liquid chromatography (HPLC; UV detection).
Standard solutions of the test material treated similary to test samples with the nitro reagent were used as basis for evaluation.
With a 52 litres atmosphere sample and an end solution volume of 25 ml, the limit of quantification for this test substance has been found to be **0.35 mg test material/m^3**.

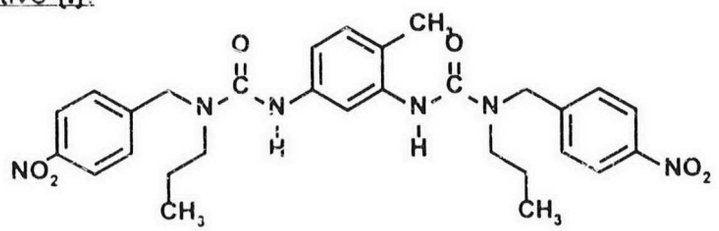
For content checks in application media the test material is placed in a solution of N-4-Nitrobenzyl-N-n-propylamine solution (nitro reagent). The isocyanate component reacts to form the corresponding urea derivative. After dilution with acetonitrile, the reaction product is quantified by high-performance liquid chromatography (HPLC; UV detection).
Standard solutions of the test material treated similary to test samples with the nitro reagent were used as basis for evaluation.
The limit of quantification for this test substance has been found to be **54.8 µg test material/ml acetonitrile**.

3. INTRODUCTION

An analytical method for the quantification of 2,4-Toluenediisocyanate from test atmospheres was developed. The TDI was the basis of the analytical concentration determinations. This work was conducted in preparation for investigations on the inhalation toxicity of this test material. The method and its validation is described in this report.

In this method, developed by N. Kuck and modified by ourselves, the test material as a vapor is allowed to react with N-4-nitrobenzyl-N-n-propylamine (nitro reagent) to form the corresponding urea compound (I), which is then determined by high-performance liquid chromatography (HPLC) with UV detection. The test material vapor is adsorbed from the test atmosphere in two series-connected tubes packed with glass powder loaded with the nitro reagent solution. The TDI urea derivative (I) was then desorbed with acetonitrile and the solution was injected, after appropriate dilution, onto the HPLC.
Standard solutions of the test material treated similary to test samples with the nitro-reagent were used as basis for evaluation.

TDI-urea derivative (I):



Investigations necessary for drafting the Standard Operating Procedure and performing the analyses were conducted in January/February 1996 at the Institute of Industrial Toxicology, Department of Toxicology of Bayer AG, D-42096 Wuppertal-Elberfeld, Friedrich-Ebert-Strasse 217-333.
The study documentation (raw data and final analytical report) has been archived in locations specified by Bayer AG, in accordance with GLP requirements.

Study-No.: T3060700

The analytical method validation (HPLC) was presented in study no. T1060636.

4. MATERIALS AND METHOD

4.2. HIGH PERFORMANCE LIQUID CHROMATOGRAPHY

4.2.1. Apparatus

High performance liquid chromatograph HP1090 equipped with **
- Autosampler
- DAD (diode array detector)
- Integration: HP 3365 DOS-WorkStation/ChemServer **

supplied from Hewlett-Packard

4.2.2. Method

Column:	LiChrospher RP 18	5 µm; L: 125 mm; ID: 4mm; Merck	**
Oven temperature:		off	
Mobile phase:	A:	50% buffer solution	
	B:	50% acetonitrile	
	gradient program:	time 3 min: 50%B --> time 6 min: 85%B	
	buffer composition:	2 ml H_3PO_4 + 4 ml TEA ad 1000 ml Milli-Q-water	
	gradient program:	time 3.0 --> %B = 50	
		time 6.0 --> %B = 85	
Flow rate:		1.0 ml/min	
Injection volume:		25.0 µl	
Detector:	wavelength:	275 nm	
	band width (BW):	4 nm	
	reference:	450 nm / 80 nm BW	**

4.3. OTHER APPARATUS

Gas measuring device (Elster) **
Mini A-Pump (P) (Leybold-Heraeus) **
Rotameter (R) **
Manometer (D) **
Needle valve (V)
calibrated thermometer for temperature measurement
calibrated barometer
Standard laboratory equipment and glassware

small adsorption tubes with ground-glass joints (L = 120 mm, ID = 12 mm)
Packing: each tube 4 g glass powder
small adsorption tubes with ground-glass joints (L = 65 mm, ID = 12 mm)
Packing: each tube 2 g glass powder

Gas tight syringes (25 µl; 100 µl; 250 µl; 10 ml ; Hamilton) **

** or equivalent

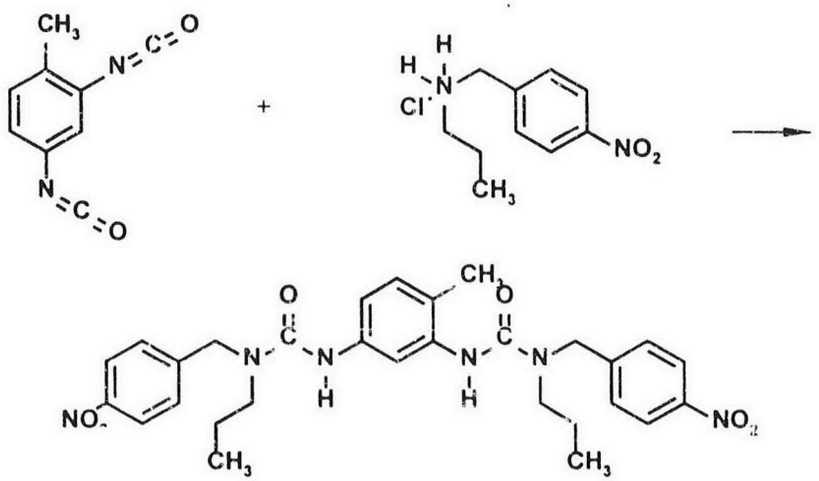
(The apparatus is regulary maintained and calibrated.)

4.4. SOLVENTS AND CHEMICALS

Acetonitrile p.a.; Merck
Deionized water (Milli-Q-water), Millipore unit
Dichloromethane p.A., Merck
N-4-Nitrobenzyl-N-n-propylammonium chloride p.A., Fa. Riedel de Haen, No. 33487
Glass powder 40/60 mesh; C. Karl, Part-No. GK 26-48004
sodium sulfate p.A., Merck
o-Phosphoric acid (85%ig), H_3PO_4 ; Merck
Triethylamine (TEA) ; Merck

4.4.1. Nitro reagent solution (absorption solution)

1.6 g N-4-nitrobenzyl-N-n-propylammonium chloride (corresponding to 1.34 g free base) is dissolved in 100 ml of deionized water and 50 ml of 1 N sodium hydroxide solution is added. A white precipitate (free base) is formed. The aqueous suspension is transferred into a 500 ml separating funnel and extracted with 250 ml dichloromethane. The organic phase is separated off, dried over sodium sulfate, transferred into a 500 ml volumetric flask, and made up to the mark with dichloromethane. This solution contains 2.7 mg nitro reagent (free base)/ml dichloromethane. The solution can be used as an absorption solution in impinger-flasks as well as for sample collection with glass powder-packed tubes, the nitro reagent serving to load the adsorbent carrier material.



empirical formula of the urea derivative: $C_{29}H_{34}N_6O_6$
The structure of the reaction product formed from TDI and nitro reagent is shown in the above equation. This urea derivative is analyzed in the HPLC (UV-detection) and is quantified, after recalculation, as free TDI.

4.4.2. Calibration standards

30-50 mg of the test material is pipetted into a 50 ml volumetric flask and accurately weighed. The flask is then brought up to volume with nitro reagent solution (concentration: 2.7 mg/ml). Comparison standards in the desired concentrations are prepared from this solution by dilution with dichloromethane or acetonitrile.

4.4.3. Structure elucidation of Desmodur T80 urea-derivative (I):

Desmodur T80 urea-derivative was synthesized to check the component identity and for the verification of the peak in the chromatograms.
Desmodur T80 is added slowly to an appropriate volume of nitro reagent solution. After the reaction has finished the solvent is evaporated and the residue of the desired product is separated, and dried overnight at 40°C. The product has a content of 98.8% (HPLC-area%). The spectra (^{1}H-NMR, MS) shown below confirm the structure unambiguously. The structure elucidation was performed in the PH-AQ-F department/Dr. Wünsche.

Figure 1a: ^{1}H-NMR spectrum (2,6-TDI)

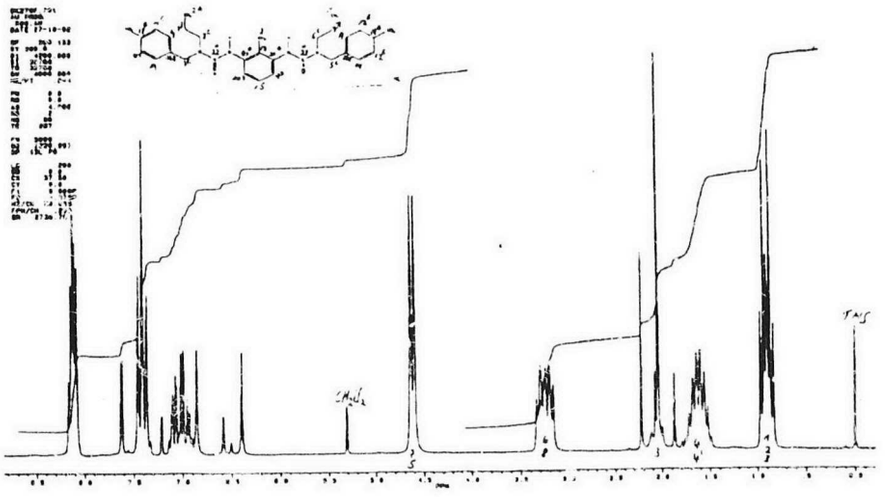
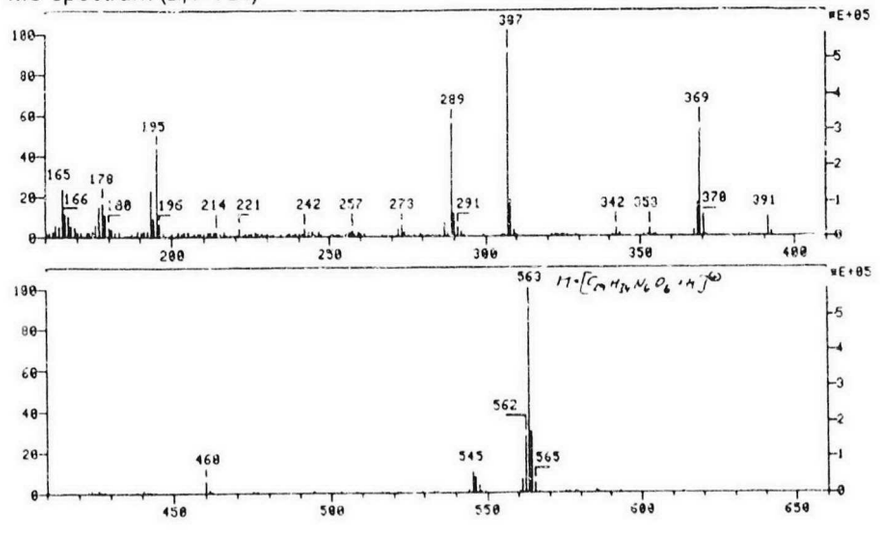


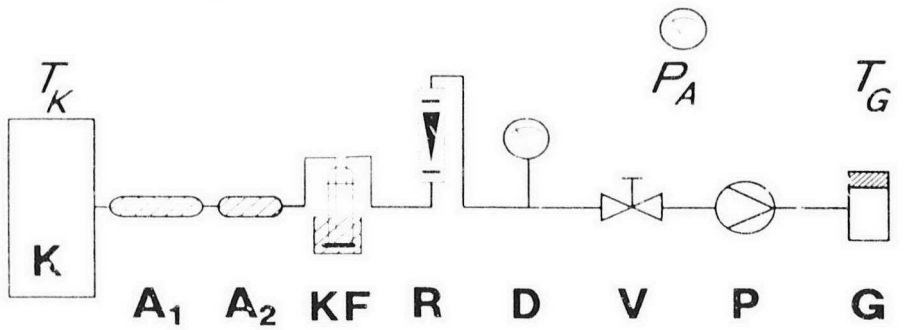
Figure 1b: MS spectrum (2,6-TDI)



5. SAMPLE COLLECTION AND PREPARATION

The surface of the glass powder in each adsorption tube is first loaded with 1 ml of the nitro reagent solution. The solvent is collected and discarded. Two series-connected adsorption tubes pretreated in the described way (A_1: 4g; A_2: 2g) are connected to the sampling apparatus (air throughput 0.5 to 1.0 l/min) (Fig. 2). The total volume of sampled air (V_X) the temperature of the gas flowmeter (T_G) the chamber temperature (T_K) and the barometric pressure (P_A) are recorded. After the end of the sample collection adsorption tubes (A_1, A_2) are mounted against the flow direction on a 50 ml volumetric flask. To desorb the urea derivative a funnel is fitted and 45 ml of acetonitrile is passed slowly through the tubes. The contents of the volumetric flask are then made up to the mark with acetonitrile. Samples of low concentration (approx. 1 mg/m^3) are eluted with 25 ml of acetonitrile. Solutions are then injected onto the HPLC after appropriate dilution.

Figure 2: Sample collection apparatus



K	Inhalation chamber	V	Needle valve
A_1	Adsorption tube; packing: 4 g glass powder	P	Pump
A_2	Adsorption tube; packing: 2 g glass powder	T_G	Temperature of Gas flow meter
KF	condenser (optional)	T_K	Temperature of chamber
R	Rotameter	P_A	Barometric pressure
D	Manometer	G	Gas flow meter

6. CALIBRATION OF THE ANALYTICAL METHOD

To set up the calibration series, test material solutions in nitro reagent solution were prepared with appropriate concentrations (see 4.4.2.). Method-specific adjustments were made on the HPLC and 25.0 µl of each calibration concentration was injected for preparation of the calibration curve.

Measurement wavelength: 275 nm (see the UV spectrum, Fig. 3)

Fig. 4 shows a typical chromatogram of these external calibration solutions. A statistically evaluated calibration curve is shown in Fig. 5. This curve was plotted by the integrator and was based upon the injected concentrations. The calibration curve was plotted anew for each analysis sequence, and deviations from this calibration range were therefore possible. All sample concentrations are always within the calibration range documented for each sample sequence. The quantitative evaluation was performed by determination and comparing the peak area of **TDI urea derivative** of the analytical solution with the peak areas of the external standard solutions.

Retention time:	**2,4-TDI urea derivative**	about 6.7 min	**conc. range:**	0.72 to 7.2 µg/ml
	2,6-TDI urea derivative	about 6.2 min		not determined

Figure 3: UV spectra of the TDI urea derivative

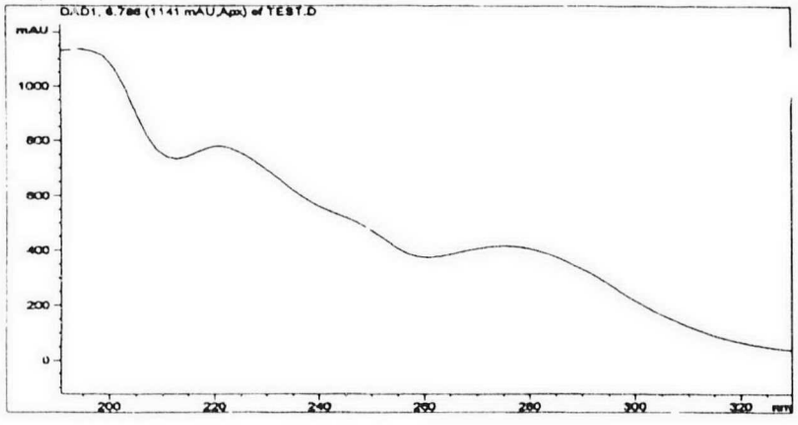


Figure 4: typical LC-chromatogram of the test substance (calibration standard)
test material concentration: 29.0 µg/ml

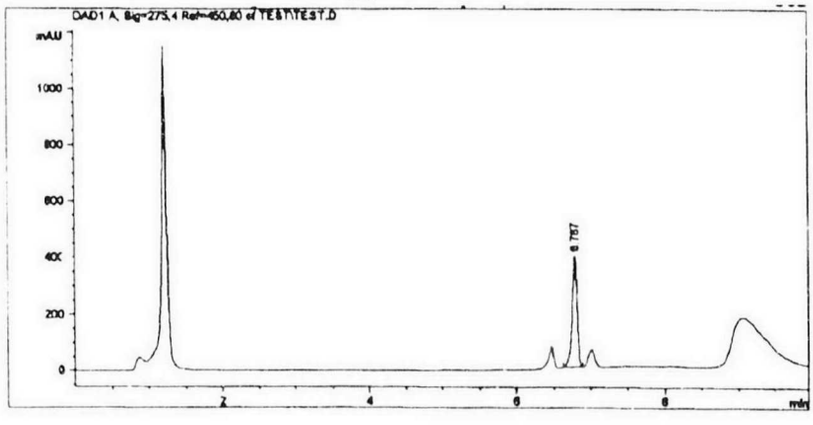
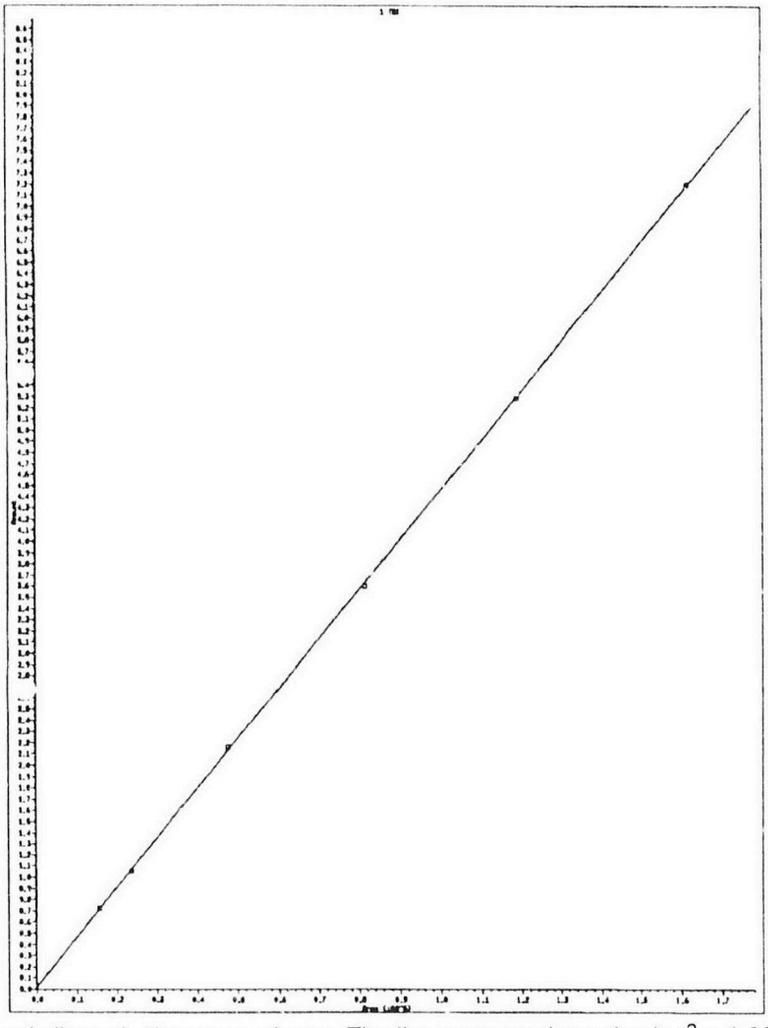


Figure 5: Calibration curve of the analytical method date: Feb. 29, 1996



The calibration is linear in the range shown. The linear regression value is $r^2 = 1.000$.

7. CALCULATION OF THE ANALYTICAL RESULTS

Each sample within a sequence was injected twice. Since the sample and standard are treated identically, the concentration results do not need to be recalculated. The integrator evaluates each sample based on the plotted external standard calibration curve (see section 6.). The results are expressed in units of µg test material/ml solution.

The test material concentration in the test atmosphere is determined from the relationship:

$$\text{mg test material} / \text{m}^3 \text{ air} = \frac{X * F}{V_X} * \frac{(273 + T_G)}{(273 + T_K)}$$

F		dilution factor (= 25/50 for undiluted analytical solution)
X	[µg/ml]	test material concentration in the analytical solution
V_X	[l]	chamber atmosphere collected volume
T_G	[°C]	temperature of the gas flowmeter
T_K	[°C]	temperature of the inhalation chamber

8. STABILITY

The stability of Desmodur T80 in acetonitrile and dichloromethane was checked at room temperature over a period of 6 days. All solutions tested were found to be stable. No decrease in concentration was observed. The chromatographic sample preparation (elution of test material from glass powder, dilution, and injection) all are inducted during the tested time frames.

9. PRECISION

The precision of this analytical method was assessed by 10 separate injections for each of two relevant concentrations of the calibration standards (raw data presented in T3060304). The area values obtained are presented in table 1. The precision of this method was found to satisfy the analytical requirements.

Table 1:

0.580 [µg/ml]	124.800 [µg/ml]
0.793	124.116
0.780	124.152
0.773	122.092
0.796	123.515
0.794	122.899
0.782	121.389
0.781	122.618
0.800	123.323
0.818	123.085
0.792	124.473
MEAN = 0.791	**MEAN = 123.166**
C_V = 1.6%	**C_V = 0.8%**

10. RECOVERY

The recovery from the adsorption material glass powder was taken from study no. T2044103.
Result: 93.0%.

11. DETECTION LIMIT

The limit of quantitation using this analytical method is 0.72 µg test material/ml in acetonitrile. With a sample collection volume of 52 litres and an end dilution volume of 25 ml, a concentration of **0.35 mg TDI/m^3** can be accurately determined.

12. LITERATURE

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End of Report

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